

BIOLOGICAL ANTIOXIDANTS

*Transactions of the Third Conference
October 7-8, 1948, New York, N.Y.*

Edited by

COSMO G. MACKENZIE

DEPARTMENT OF BIOCHEMISTRY
CORNELL UNIVERSITY MEDICAL COLLEGE

Associate Editors

RICHARD H. BARNES

SHARP AND DOHME, INC.

LESLIE HELLERMAN

THE JOHNS HOPKINS SCHOOL OF MEDICINE

KARL E. MASON

SCHOOL OF MEDICINE AND DENTISTRY,
UNIVERSITY OF ROCHESTER

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565 PARK AVENUE, NEW YORK, N. Y.

Published by the

JOSIAH MACY, JR. FOUNDATION

565 Park Avenue, New York 21, N. Y.

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WITH THE accelerating rate at which new knowledge is accumulating and with the increasing recognition that nature is of one piece, it becomes evident that the continued isolation of the several branches of science from one another is a serious obstacle to scientific progress.

Nowhere in science is the need for "combined operations" more evident than in medicine. Today, to be effective, medical research and practice must embrace data from all the disciplines including nuclear physics at one end of the spectrum and cultural anthropology at the other, for advances in one field are frequently dependent upon knowledge derived from quite another discipline.

Although the fertility of the multi-discipline approach is thus recognized, universities, scientific societies and journals have not yet made adequate provision for channels of interdisciplinary communication.

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In order to share with a wider group of investigators and students the essential quality of these conferences the informal nature and tempo of the discussions in so far as possible are preserved in the published transactions.

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Third Conference on Biological Antioxidants

PAUL GYÖRGY, *Chairman*

Department of Pediatrics, University of Pennsylvania School of Medicine
Philadelphia, Pa.

COSMO G. MACKENZIE, *Secretary*

Department of Biochemistry, Cornell University Medical College
New York, N. Y.

STANLEY R. AMES

Research Laboratories, Distillation Products, Inc.
Rochester, N. Y.

RICHARD H. BARNES

Medical Research Division, Sharp & Dohme, Inc.
Glenolden, Pa.

C. A. BAUMANN

Department of Biochemistry, University of Wisconsin
Madison, Wis.

W. G. CLARK

The Scripps Metabolic Clinic
La Jolla, San Diego, Calif.

W. MANSFIELD CLARK

Department of Physiological Chemistry, The Johns Hopkins School of Medicine
Baltimore, Md.

LOUIS F. FIESER

Department of Chemistry, Harvard University
Cambridge, Mass.

LESLIE HELLERMAN

Department of Physiological Chemistry, The Johns Hopkins School of Medicine
Baltimore, Md.

KENNETH C. D. HICKMAN

Consultant, Arthur D. Little, Inc.
Boston, Mass.

RALPH T. HOLMAN

Department of Biochemistry and Nutrition, Agricultural and Mechanical College of Texas
College Station, Texas

ALBERT L. LEHNINGER

Departments of Surgery and Biochemistry, The University of Chicago
Chicago, Ill.

WALTER O. LUNDBERG

The Hormel Institute, University of Minnesota
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KARL E. MASON

Department of Anatomy, University of Rochester School of Medicine and Dentistry
Rochester, N. Y.

HENRY A. MATTILL

Department of Biochemistry, The State University of Iowa
Iowa City, Ia.

ROLAND K. MEYER

Department of Zoology, University of Wisconsin
Madison, Wis.

L. MICHAELIS

Rockefeller Institute for Medical Research
New York, N. Y.

F. W. QUACKENBUSH

Department of Agricultural Chemistry, Purdue University
Lafayette, Ind.

HAROLD P. RUSCH

McArdle Memorial Laboratory for Cancer Research, University of Wisconsin
Madison, Wis.

JOSEPH SEIFTER

Wyeth Institute for Applied Biochemistry
Philadelphia, Pa.

WILLIAM C. STADIE

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Philadelphia, Pa.

C. E. SWIFT

Southern Regional Research Laboratory
New Orleans, La.

A. V. TOBOLSKY

Frick Chemical Laboratory, Princeton University
Princeton, N. J.

ARNOLD WEISSBERGER

Synthetic Organic Research Laboratory, Eastman Kodak Co.
Rochester, N. Y.

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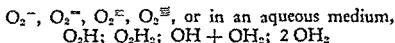
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THE MECHANISM OF AUTOXIDATION AND THE ACTION OF AN ANTIOXIDANT

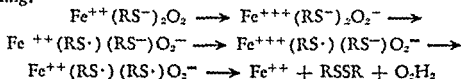
L. MICHAELIS

The Rockefeller Institute for Medical Research

THE INERTIA of molecular oxygen with respect to its thermodynamically inherent oxidizing ability can be attributed to the "principle of compulsory univalent oxidation-reduction." Every oxidation or reduction must proceed in successive univalent steps each involving the transfer of a single electron. These steps are, for O_2 , the following:



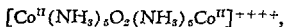
The high energy to produce the free radicals O_2H and OH represents a barrier for the reduction of O_2 , or the activation energy for the reaction $O_2 \longrightarrow O_2H_2$, and $O_2H_2 \longrightarrow 2H_2O$. If oxygen does oxidize, there must be a mechanism to overcome the activation energy. How this can be achieved, may be shown for the example of the oxidation of cysteine, RSH , to cystine $RSSR$, by molecular oxygen. This process requires a catalyst, e.g., a small amount of an iron salt. To understand the mechanism, replace iron by cobalt. Then, a cobaltous bi-cysteine complex is formed in which not all of the six coordination places are occupied. They may be filled either with O_2 or Co . In the latter case, a well-characterized complex is formed, containing the cobaltous ion as center. However, when O_2 is bound, an intra-molecular redistribution of electrons takes place, reducing O_2 , and oxidizing the cobaltous ion to the cobaltic state. This is the end of the reaction. With Fe instead of Co , because of the ease at which iron can be reversibly changed from Fe^{+++} to Fe^{++} , the analogous process does not stop at the corresponding stage. If the complex is symbolized as $Fe^{++}(RS^-)_2$, then a chain of single-electron transfers will take place after the addition of O_2 to this complex, the stages being:



where $RS\cdot$ is a radical. There is an alternate possibility that O_2 forms a bridge in a binuclear complex. If so, O_2 would be reduced directly to $2 H_2O$. The reason O_2 can be reduced intramolecularly within the complex is the fact that O_2 as a ligand in the complex has an electronic structure essentially different from that of free O_2 . The latter is in the triplet state, paramagnetic. Within the complex it is in the singlet state, diamagnetic. The situation is comparable to the contrast of free H_2 and H_2 bound to palladium, where it behaves almost as though it consisted of two separate H atoms.

The precursor of oxidation by O_2 ("autoxidation") is the formation of an "oxygenated" complex; subsequent electron redistribution may lead to true oxidation. The hypothesis of an oxygenated complex as precursor to oxidation is supported by the fact that in a number of cases the oxygenated complexes have a sufficiently long lifetime before intramolecular oxidation takes place, and can be prepared as well-defined chemical entities which can be reversibly oxygenated or de-oxygenated according to the oxygen pressure, the change being manifested by a change in color.

The only known case among iron complexes is the well-crystallized compound, $[Co^{II}(NH_3)_5O_2(NH_3)_5Co^{II}]^{++++}$, which reacts with O_2 to form free NH_3 and the well-crystallized compound,



which is a perfectly stable compound in the solid state, but releases all its oxygen suddenly when dissolved in acid (demonstration). When dissolved in excess of NH_3 , the oxygen can be reversibly attached or detached according to O_2 -pressure; however, very gradually, a true oxidation to cobaltic complexes takes place. A large group of cobalt complexes which can be reversibly oxygenated, has been prepared by Calvin and his associates, and similar cases, according to D. Burk and his group, are represented by cobalto complexes of certain amino acids, especially histidine. Here always a relatively stable oxygenated complex is the precursor of a slow subsequent irreversible oxidation process.

The autoxidation of unsaturated fatty acids is another reaction in which an oxygenated compound is the precursor of a true oxidation. I need not discuss before this audience what has been known about the matter. The hydroperoxides of the unsaturated fatty acids have been prepared as relatively stable molecular entities and have been recognized as precursors to the oxidation proper of these acids. I do not think that the mechanism of this process

Mechanisms of Autoxidation

has been clarified yet to the same extent as the iron catalysis described just now. This fact will make it even more difficult to interpret the action of the antioxidants in detail. However, one may utilize our present knowledge in those other fields for some suggestions. I would request that you take these suggestions as presented by somebody who has no experience of his own in this field of autoxidation, and has been asked for suggestions according to his experience in related fields.

First of all, one should keep in mind that the antioxidants may be represented by two classes of organic compounds, one represented by hydroquinone, which can be reversibly oxidized to quinone, and the others by tocopherol, which cannot, anyhow not in a reversible way, be oxidized to a quinone. That quinone which can be obtained from tocopherol on oxidation, is not a reversible oxidation product since the phytol side-ring is irreversibly opened on oxidation. This fact suggested the idea that the bivalent oxidation of hydroquinone to quinone may not be involved either in the mechanism of its antioxidant property. What is common to hydroquinone and tocopherol seemed to me the possibility that both may be reversibly oxidizable to a free semiquinone radical. That this is the case for hydroquinone will not be doubted by anyone any longer. Such a semiquinone can be prepared, under suitable conditions, in an appreciable concentration, in equilibrium with the hydroquinone and the quinone. Such is the case, for instance, for the semiquinone derived from duroquinone or phenanthrenequinone in an alkaline solution, where its existence has been demonstrated in our laboratory both by potentiometric and magnetometric methods. In the case of tocopherol, nothing has been known so far about the existence of a semiquinone nor can one expect it to be capable of existence at a concentration high enough to be readily detectable. Since the corresponding quinone may be said to be utterly unstable insofar as it rapidly undergoes an irreversible reaction through the opening of the phytol ring, the semiquinone cannot accumulate either to any noticeable concentration if equilibrium is to be established. However, there is a method to prevent the establishment of equilibria: to work at very low temperature in a rigid, not fluid solvent.

This method was devised by G. N. Lewis and is as follows. The substance in question is dissolved in a mixture of alcohol, ether, and pentane, which, when cooled in liquid air, forms a homogeneous glass instead of crystallizing. This rigid solution is irradiated through a quartz window in the Dewar flask, with ultraviolet light. Hereby, two effects may be produced; either an electron is

reduced state. Ball measured the half-life of the oxidant at pH 7 with our rapid flow technique. It is six one-hundredths of a second. The hemoglobin system is definitely positive to that of the cell and although a little methemoglobin is found in the blood, wherever methemoglobin comes in contact with a process of metabolism it's restored to hemoglobin. One could go on almost indefinitely to support the contention that the observed potentials of cells are significant because they correlate with the states of certain known oxidation-reduction systems found in living things.

This apparent potential of cells in aerobiosis is very far from the point where oxygen exhibits its punch. Furthermore, a great many of our disinfectants are oxidation-reduction systems which tend to pull the systems of the cell up into this upper region. So when we say that any catalyst found in the living body activates oxygen, we cannot say, I think, that it activates the oxygen-water system in the sense that platinum and certain biological catalysts activate molecular hydrogen.

Now, the potential of the hydrogen peroxide system is down in this region (pointing) and we know that hydrogen peroxide is concerned in certain processes in the living cell.

The questions I want to ask Dr. Michaelis are these. First, does he not agree that in oxidation, biological oxidation, the cell does not activate oxygen in the sense that platinum activates hydrogen. In the cell oxygen is put through particular channels; its action is directed to highly specific compounds. And now comes the question of the thermodynamic aspect concerning which I'm a bit puzzled. Are the potentials of the systems mentioned by Dr. Michaelis reflected in the observed cell potentials I've mentioned?

Michaelis: I think Professor Clark is right in saying that it may be misleading to speak of any "activation of oxygen." Oxygen is used as an oxidizing agent in respiration, not directly, but in the following indirect way: oxygen combines with cytochrome oxidase in the same way as CO combines with it, by occupying a co-ordination place of the iron atom of that enzyme. But, whereas the CO-complex remains as such, the O_2 -complex rapidly undergoes an intramolecular electronic rearrangement such that O_2 is reduced and Fe^{++} is oxidized to Fe^{+++} . It is this Fe^{+++} which oxidizes cytochrome c. The latter cannot be oxidized directly by O_2 . As regards the problem of why O_2 can be reduced when forming a part of the complex, whereas it cannot be reduced when in the free state, one has to consider that the electronic structure of O_2 in the free state is not the same as in the combined state. In the free state, O_2 is in its ground level of energy, a para-

magnetic molecule, with two unpaired electrons, a state called a "triplet state" by spectroscopists; whereas in all cases where any oxygenated complex is stable enough for magnetic measurements the bound O_2 has been shown to be diamagnetic. This fact shows that bound O_2 is not the same as free O_2 , and obviously the bound O_2 is easier to reduce than is free O_2 , but as hydrogen gas, when bound to palladium, is easier to oxidize than is free H_2 ; in the bound state H_2 behaves as though it consisted of two almost separated H-atoms. This mechanism of what, loosely, may be called "activation of oxygen" has no relation whatever to the oxidation potential of that almost imaginary, scarcely realizable galvanic oxygen-hydrogen cell. In respiration, O_2 does not react at all with hydrogen, but with iron.

As regards the oxidation potentials as studied first by Gillespie in systems with living organisms and elaborated so carefully by Professor Clark and his associates, they are, although of great significance in various respects, not yet comparable to potentials obtained under conditions of equilibrium. They are not determined by thermodynamics alone but depend on rates. A steady state of the complex system may be maintained if there is a constant supply of oxygen and a constant rate of elimination of the end-products of respiration. The steady state depends on the rates of all the elementary electron-transfer processes which are involved in bringing about the steady state. However, the fact that the potentials, measured in the presence of oxygen (or in presence of any other "ultimate electron acceptor" such as nitrate, in the case of anaerobic organisms) have no simple thermodynamic significance, should not be interpreted by saying that such potentials are meaningless. Quite to the contrary, they are important characteristic properties of the respiring systems.

György: Professor Clark?

Mansfield Clark: Dr. Michaelis, in your abstract you say, and I quote, "is activated so as to develop its thermodynamically inherent oxidative power." Now, I was hoping that, having stressed some of the states of oxygen and hydrogenated oxygen compounds, you might tell us whether the observations to which I refer reflect the potentials of the systems you mention. I agree with you thoroughly that my observations do not concern an equilibrium state of reversible systems. I've never held that view. But does the potential down there in the middle reflect the production of any of the systems you've mentioned? We know the potential of the hydrogen peroxide system, but do you have any information on the potentials of these other systems?

Michaelis: You mean the normal potentials of the oxygen system in its different levels of oxidation-reduction. My answer is this. Some years ago we had a conference at the New York Academy of Sciences in which Dr. Gorin discussed those potentials and gave approximate values for the normal potential of the couple, O_2 , O_2^- ; for O_2^- , O_2^{2-} , etc. I think those normal potentials have no bearing in respiration. First of all, a normal potential is a potential holding for some suitably chosen standard condition of the molecular species concerned, say normal concentration, or 1 atmosphere pressure, etc. No standard conditions for such an unstable species as O_2^- can be defined. Furthermore, the energy relationships between O_2 and O_2^- etc. in the free state are certainly quite different from those in the state as they are utilized by the enzymes, namely, as components of a complex compound. Furthermore, the reactions $O_2 \rightarrow O_2^-$ etc. take place intramolecularly within the complex; the oxidation-reduction system is entirely an intramolecular one; one part of the complex molecule is oxidized at the expense of another part of the same molecule. It would not be adequate to speak of a normal potential of such an intramolecular redox system.

György: Any further discussion?

Lehninger: I wanted to ask a question very closely related to what you've just been discussing. I'm interested in biological oxidations of the organized variety, let's say Krebs's tricarboxylic acid cycle and the electron transport which accompanies that through the respiratory enzyme. One of the big questions is of course the mechanism by which the energy liberated during the passage of a pair of electrons from substrate to oxygen is converted into phosphate bond energy. Now one of the big questions that arises is this. Is there any possibility that the energy released on interaction of oxygen with the respiratory enzyme itself, the $Fe^{++} \rightarrow Fe^{+++}$ transformation, is of sufficient magnitude to account for the formation of one high energy phosphate bond which requires some twelve kilocalories. Furthermore, can you visualize any possible mechanism for the transformation of energy liberated on the oxidation of the reduced respiratory ferment by oxygen into phosphate bond energy? That oxidation step represents one of the large potential drops during the course of electron transport.

Michaelis: Nobody can answer those questions in detail, but there is every reason to assume that the free energy change involved in certain steps of the chain of electron transfers is compatible with the energy necessary to form a phosphate bond provided there is a mechanism to utilize the energy in the proper way

instead of wasting it as heat. Oxygen has only the role (although a most important role) of starting the chain of electron transfers by oxidizing cytochrome oxidase to the ferric state. In the Krebs cycle, molecular oxygen plays no recognizable role. Your real problem is as to the mechanism of the storage of the free energy in the form of a phosphate bond.

Lehninger: Perhaps I didn't phrase my question just right. What I was more interested in was the guess as to the amount of energy liberated during the oxidation of reduced cytochrome oxidase—whether that could be approximated even though you don't know exactly what the oxidation-reduction potentials of cytochrome oxidase are.

Michaelis: We know it can oxidize cytochrome. Is that sufficient for you that we know the chain goes on?

Lehninger: Granted that it goes on, I'm just wondering what is the magnitude of the energy released during the reaction between molecular oxygen and cytochrome oxidase?

Michaelis: I have no figures. I don't know. It cannot be very much because the root processes are divided in many steps.

György: May I ask Dr. Michaelis whether the demonstration of a tocopherol radical implies that tocopherol will act in the living organism through chain-reactions? This brings us to our perennial question: Do chain reactions occur in the living body?

Tobolsky: Theoretical calculation leads to the thought that there should exist at a given temperature a maximum rate of oxidation, a rate at which the chain length of the chain reaction is unity.

We made several observations of rubber oxidation accelerated by light and increased the light intensity so the rate got faster and faster and we noted that beyond a certain light intensity there was no further acceleration of oxidation. An interesting sidelight to this is that when you have a very high intensity of light, you can put a half sector in front of the light source and if it is rotated at a sufficiently fast speed, the reaction with the half sector present is exactly the same rate as though the sector were not there at all. At sufficiently high temperatures the maximum rate of oxidation apparently is reached thermally, for at sufficiently high temperatures there is no increase in the rate of oxidation in the presence of light. Certain activators, such as those which activate decomposition of hydroperoxide, will also act in the same way as light; i.e., they will accelerate the oxidation rate up to a certain maximum rate which cannot be exceeded at a fixed temperature. I was wondering if this phenomenon of a maximum rate of oxidation has

any biological counterpart.

Weissberger: I don't know of a biological counterpart, but we have a similar phenomenon in the autoxidation of trimethyl hydroquinone. Trimethyl quinone is a catalyst of that oxidation, but if we add increasing amounts of the quinone to the hydroquinone, we reach a saturation of the catalysis and you can add any amount of quinone after that without catalytic effect.

Tobolsky: Do you know of any examples of a photo-saturation?

Michaelis: Is there a photo-saturation in the hydrogen chlorine explosion?

Tobolsky: No, the rate in this case varies as the square root of the light intensity.

Michaelis: And the length of the light. The duration of the illumination. Or is too fast?

Tobolsky: In most cases of photo chain reactions the rate is proportional to the square root of light intensity. If you use a rotating half sector, the rate under certain conditions is one-half of the rate in absence of sector and under other conditions it is .717. I would like to enquire whether anyone knows of any other cases where in a reaction accelerated by light the interposition of a half sector has *no* effect on rate.

Lundberg: At the first Conference on Biological Antioxidants, Dr. Taylor classified antioxidants into two types: (1) Antioxidants which act by interrupting reaction chains. (This type of inhibition has been shown by Bolland and ten Have to operate in the autoxidation of methyl linoleate in a single phase system containing a phenolic type of inhibitor. In this system the only reactants involved in the formation of peroxides are methyl linoleate and oxygen, and free radicals formed from them. The chain mechanism is carried on by means of free radicals and is interrupted only when two free radicals react, or when a free radical reacts with the antioxidant.) (2) Antioxidants which act by removing or inactivating a prooxidant.

The question which arises here is whether biological antioxidants may not fall almost exclusively into this second category. In the case of biological oxidation systems, in addition to the oxidizing substrate and oxygen, there are present enzymes or other substances that play a role in the oxidation mechanism. In this case, the biological antioxidants presumably may act by removing or inactivating special types of prooxidants such as the enzymes or other materials that play an accessory role in biological oxidations.

I should like to ask Dr. Michaelis whether he believes that biological antioxidants in general should be classified in the second

category, or whether he knows of any cases of biological oxidations where the antioxidant falls into the first category, i.e., where the antioxidant acts by interrupting reaction chains.

Ames: There appears to be an inverse relationship between the biological potency of the tocopherols and their antioxidant ability for vitamin A, carotene and similar substances. This is also true of the ease of oxidation. In other words, δ -tocopherol, the most active antioxidant, is the least potent biologically and α -tocopherol, which is the most potent biologically, is the most readily oxidized.

György: Possibly Dr. Mattill may wish to comment.

Mattill: As far as I know, there has not been any satisfactory parallelism.

György: How about the high oxygen consumption of the muscles of vitamin E-deficient animals?

Mattill: Studies of the enzyme systems involved have not yet provided any consistent explanations. The secret will likely be revealed by the demonstration of alternate pathways of oxidation.

György: Dr. Mackenzie?

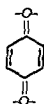
Mackenzie: The question brought up is one that Dr. Taylor discussed at some length and really is this: whether, in the oxidation of fatty acids *in vivo* to give the hydroperoxide, tocopherol blocks this reaction after the radical has been formed and becomes propagating, or whether it does this by inhibiting an enzyme. Now, I would mention a possibility which seems to me to make the question much more difficult, i.e., that the antioxidants and the enzyme are intimately associated in the cell. We know of such examples with enzyme-coenzyme systems. If this is the case we will have great difficulty in determining whether the antioxidant is blocking an enzyme or is stopping the reaction at its inception as soon as the first radical is formed so that the chain cannot be propagated. In other words, the oxidation would not be effective with either of the two mechanisms, and you would be put to it to say whether the antioxidant is inhibiting the pro-oxidant or is reversing its action at every single step. Precisely where in the living cell do the antioxidants exist and what is their association with the oxidizing enzymes? This problem in chemical cytology is extremely important.

Hickman: I think there is a point to bear in mind concerning the words "in vivo" and "in vitro." With "in vivo" surely we have to define two stages, a primary and a secondary. The primary stage is the one that perhaps would involve enzyme activation or interaction. The other one is really a kind of "in vitro-in vivo" status. It represents a great mass of reactive material held in the

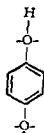
organism which is not going to interact with the enzyme at this moment at all and yet it is amenable to gaseous or chemical oxidation which proceeds quite separately and apart from what one might call the vital processes. The organism, and hence the science of biochemistry is perpetually dealing with this wastage. The waste products of these secondary reactions are complicating and going on side by side with the primary reaction which is the true "in vivo" oxidation or reduction or inhibition.

György: If there are no more questions on the first paper, we shall go to the second paper. Dr. Weissberger?

Dr. Weissberger: I would just like to make sure that I understood Dr. Michaelis properly. This is a quinone



and if we add a hydrogen atom, we obtain a semiquinone.



Oxygen is analogous to a quinone, and if we add it to a hydrocarbon radical, the complex corresponds to a semiquinone. Two molecules of a semiquinone disproportionate into one molecule of the reduced form and one molecule of the oxidized form. Is it the oxido-reduction between the two compounds that regenerates oxygen and the hydrocarbon and breaks the chain?

Michaelis: This is a very reasonable question. My answer would be this. The tocopherol radical can exist only in a very low concentration. In order to bring about any kind of interaction of one radical molecule with another of the same kind, a bimolecular collision is necessary. At the low concentration of the radical, it would rather collide with any other molecular species with which it could react than with another molecule of its own kind. So, inactivation of the radical due to dismutation will be a highly improbable, rare event.

Wienberger: That would explain why you get a lot of chain breaking before you get some termination.

Ames: I would like to ask Dr. Michaelis a question concerning the term antioxidant. Let us refer for a moment to the subject of inorganic oxidation. A number of years ago oxidations were considered to involve molecular or atomic oxygen. Then the term became generalized as we now consider it as an oxidation reaction involves the loss of an electron. Dr. Michaelis has defined the term antioxidant in his summary as "a substance which prevents oxygenation." Is that strictly true or can we not logically extend the term antioxidant to cover those substances which diminish or prevent the loss of electrons? For example, you would then consider that malonic acid is an antioxidant when it inhibits the oxidation of succinate by the succinate oxidase system.

Michaelis: In fact, one should distinguish two kinds of antioxidant effects. An antioxidant may either prevent the oxygenation, the necessary precursor of oxidation, or it may interrupt the course of events after oxygenation has taken place. In the case of unsaturated fatty acids, it seems, if I understand it right, that the antioxidant effect is exhibited after oxygenation has taken place. In this case, the antioxidant does not inhibit oxygenation, but prevents or changes the secondary reactions which the oxygenated molecule will undergo.

tially the same oxidation-reduction potentials. Protein-binding very definitely is a highly important factor (6). A typical member of the series is practically insoluble in an aqueous buffer of pH 7 but dissolves abundantly in human plasma (e.g., 100-150 mg. per 100 cc.) to give a rich red solution. The quinone cannot be disengaged from plasma proteins by dialysis; but it is not bound by primary linkage, because addition of alcohol or alkali denatures the protein and liberates the naphthoquinone. Experiments with isolated protein fractions showed that albumin is the chief protein responsible for drug binding. We found further that the inhibitory action of a given naphthoquinone on the respiration of a suspension of parasitized duck erythrocytes in duck serum is markedly suppressed by the addition of human serum. Further comparisons showed that the order of antagonism to drug action exerted by plasma proteins is: human > chicken > duck, which corresponds to the order of effective doses in the last two species. We found further that in several of the discrete series of compounds studied definite alternation from odd- to even- carbon homologs is observable in the molar antirespiratory activities. Determinations of the relative susceptibility to protein antagonism also revealed alternation effect, in the sense that the homologues of higher antirespiratory power are the ones that are the most susceptible to deactivation by protein binding. A competition for naphthoquinone appears to exist between plasma proteins and a respiratory enzyme, and the odd or even character of the side chain seems to promote firmness of binding to both proteins. Therefore the drug action of the naphthoquinones appears to depend upon the ability of the substance to combine with, and deactivate, an enzyme necessary to the life of the parasite-containing cell. The site of action is not clear, for no detectable amount of naphthoquinone diffuses into the red cell. Since hemoglobin does not exert an antagonism to the antirespiratory action of the naphthoquinone, the effect upon respiration may be due to the diffusion into the cell of an amount of drug too small to be detected by the methods used.

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*A series of 17 papers from Harvard University, Abbott Laboratories, The University of Tennessee, and the Squibb Institute for Medical Research.

DISCUSSION

Gjörgy: Is there any discussion on Dr. Fieser's presentation on naphthoquinones and the inhibition of different enzyme systems?

Lehninger: Was there any attempt to find out whether these compounds would inhibit specific enzymes of respiration like cytochrome oxidase?

Fieser: Some experiments were done with the succinate oxidase system by Ball and Anfinsen.

Hellerman: E. G. Ball and co-workers concluded that action probably is below cytochrome c and above cytochrome b in the respiratory chain of catalysts.

Lehninger: I was just wondering what effect these compounds would have on the respiration of normal tissues, whether they would also be inhibitory?

Fieser: I think not. No particular effects have been observed that seem attributable to such an action.

W. G. Clark: What was the nature of the binding to albumin?

Fieser: Well, it isn't through any primary valence link, I'm sure, because the quinone can be recovered by adding alcohol to denature the protein.

W. G. Clark: Through some electro-kinetic source?

Fieser: The way cholesterol or other lipid is bound to albumin, I suppose. In this case we noticed that the apparent acidity constant, the apparent pK in the presence of albumin, is about two pK units more acidic than in the absence of the protein.

Hellerman: Naphthoquinones of this type appear not to oxidize protein sulfhydryl groups appreciably.

Fieser: No, I don't think they do at all.

Rusch: I think the work of Drs. E. C. Miller and J. A. Miller of our laboratory concerning the binding of the carcinogenic dye 4-dimethylaminoazobenzene with proteins of the rat liver is pertinent to this discussion. They found a firm complex between the azo dye and liver protein which was broken only after prolonged alkaline hydrolysis. Although the azo dye was found in an unbound form in other organs, it formed this complex only with the proteins of the organ in which neoplasms were induced (the liver) and only in the livers of those species of animals that developed hepatic cancer. Of further interest is the fact that the dye did not combine with the protein of the cancer cells. This indicated a different protein composition of liver tumors as compared to that in cells from normal livers.

Michaelis: Have you any experience about the autoxidizability of the hydroquinone of this series?

Fieser: No, but I think it would be comparable to the hydroquinone of phthiocol.

György: Dr. Stadie, did you want to ask something?

Stadie: Yes, is it possible that a reversible effect is produced by these compounds on your cells?

Fieser: We conducted an experiment bearing on this point in a sense, in which we allowed the red cells to be inhibited by the quinones for, say, one hour and then we added protein to combine with the quinone and remove its inhibition. When the quinone acted on the red cells for an hour we saw no subsequent effect on the rate of oxygen uptake but when it acted for about two or three hours then the cells showed evidence that the respiration was definitely impaired. This would indicate that the quinone exerts a destructive action by upsetting the respiration of the cells.

György: Any further questions?

Seifter: Was there some toxic manifestation of the compounds?

Fieser: Well, they produced some gastrointestinal upset in some individuals and some of them produced malaise but not all. This one, for example, M-2350 if given intravenously produces no symptoms in dosages of two or three grams a day for several days. We give it in solution in pyrogen-free gelatin, which seems to have an action like albumin. This seems to be a very effective way of administration and has led to no symptoms at all so far.

Seifter: How about blood counts and the blood picture in general?

Fieser: There is no effect on the NPN.

György: You mentioned that vitamin K activities were measured.

Fieser: Well, yes, these naphthoquinones when given in massive doses to rats and mice produce a hemorrhagic condition; that is, they seem to counteract the action of vitamin K. But I don't believe the effect shows up in therapeutic doses or gives rise to abnormal prothrombin levels in human subjects.

Lehninger: Could you prevent it with vitamin K?

Fieser: The effect can be counteracted with vitamin K, the natural vitamin, but not with methyl-naphthoquinone.

György: That's very interesting.

Fieser: Yes, the situation is like that in dicumarol therapy; the natural vitamin is effective and methyl-naphthoquinone doesn't work or doesn't work as well.

Stadie: Is the oxygen uptake on normal duck cells any different than the ones you took?

Fieser: We never investigated this point. The oxygen uptake of normal duck cells is not very great.

György: Any further questions?

Seifter: I don't recall the work done on this even and odd side chain effect and whether it had to do with red cell respiration, but a similar effect was noticeable in other antimalarial series. I wonder if you recall.

Fieser: It doesn't seem to me that we had enough comparable series of homologues.

Seifter: I think in the Plasmochin series it was alternated.

György: Is there any reason for that?

Seifter: They were attempting to determine diffusibility into the red cell. The theory at the time was that antimalarial activity was dependent upon the penetration of the red cells. The following is quoted from *Chemotherapy of Malaria* by J. H. Williams, published by Lederle Laboratories, June, 1941, pp. 117-120: "Compounds having chains containing an odd number of carbon atoms showed higher activity than the even numbers most closely related thereto. This alternation was found to exist by the Russian School including Magidson et al (1933) (1935), Kritchevski et al (1935) working with *Pl. praecox*, as well as by Bover et al (1934) and Altman (1935) using *Pl. relictum* in Canaries and Fournneau et al (1933) who worked with *Haemoproteus* infections in Java Sparrows." The illustrations on pp. 118 and 119 clearly demonstrate the alternation phenomena.

Fieser: There is one other effect of these compounds that I men-

tioned to Dr. Lehninger. A. P. Richardson found from his studies in ducks that a given naphthoquinone will act in synergism with plasmochin. He found that one-tenth the effective dose of naphthoquinones plus one-tenth the effective dose of plasmochin produces one hundred per cent cures. This points to another possible application of these quinones in therapy, if they are not satisfactory alone. The plasmochin compounds are curative but dangerously toxic; the use of naphthoquinone would make it possible to cut down the dose.

Hellerman: You have a similar "classical" situation in quinine-plasmochin therapy.

W. G. Clark: How do the aminoquinolines work?

Fieser: I do not believe the mechanism of action is known, but they undoubtedly act by a mechanism different from that of the naphthoquinones. These are rather unique chemotherapeutic agents; they are nitrogen-free and are very simple carbon-hydrogen-oxygen compounds. The fact that the naphthoquinones potentiate plasmochin seems to me to suggest that the two drugs act by different mechanisms. Probably one inhibits one enzyme, the second inhibits another.

Some mention of the inhibition of other enzyme systems by other naphthoquinones may be of interest. René Dubos found that some of our hydroxyalkylnaphthoquinones effect dramatic inhibition of tubercle bacilli in culture media, but that the effect is very strongly counteracted by plasma albumin. Although some suggestion of parallelism between antitubercular and antimalarial activities was noticed, an extension of the studies conducted with collaboration of the Merck Institute of Therapeutic Research has led to the development of hydroxyl-free naphthoquinones that have no antimalaria activity but show much higher *in vitro* potency against the tubercle bacillus. The experiments have not gone beyond the *in vitro* stage. Still other naphthoquinones that we have synthesized show both *in vitro* and *in vivo* activity against the schistosome (experiments by Dr. Bueding at Western Reserve); they act by inhibiting anaerobic glycolysis. Still other of our naphthoquinones have been found by Dr. Nachmansohn at Columbia to be effective in the inhibition of choline esterase.

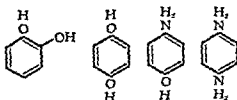
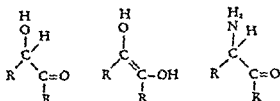
Weissberger: Hydroquinone itself is a relatively harmless compound as far as its allergenic action on the skin is concerned. When we made hydroquinone lipidsoluble by introducing side chains we ran into effects which are very similar to those of poison ivy. This is another example of a series of compounds in which the chemical properties are changed but little, however, with the change in solubility, i.e., the distribution between water and ether, a great change in physiological effects results.

AUTOXIDATION OF ALPHA-KETOLS, ENEDIOLS, AND HYDROQUINONES: QUINONE CATALYSIS AND INHIBITION

W. WEISSBERGER

Eastman Kodak Co.

WE HAVE studied the alkali-catalyzed autoxidation of the following types of compounds:



There are certain similarities in the mechanisms of these reactions, and for the present purpose, the presentation may be limited to the compounds without nitrogen. Most of our experiments have been run at a fairly high pH, and our results may therefore have no direct biological significance unless enzymes are able to affect weak acids with results similar to those produced by hydroxyl ions.

The apparatus for measuring the rate of autoxidation (1) consists of a vessel connected to a burette and provided with a dropping funnel. The vessel is shaken by an eccentric mechanism. There is a bypath from the shaking vessel to the dropping funnel. Let us assume that we measure the rate of oxidation of an alkaline benzoin solution. A neutral benzoin solution is put in the bottom part of the reaction vessel and a solution of alkali into the top

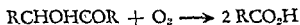
part. The system is then swept with oxygen or left filled with air and thermostated. After the temperature has reached equilibrium, the tap at the dropping funnel is opened, and the shaking mechanism is started. Readings at the burette can be taken from the very beginning, because the secondary path between the two vessels ensures that no change of volume will occur when the tap is opened. The readings are plotted against the time in the usual way and rate constants are calculated. The apparatus is very efficient in the investigation of rather fast reactions, particularly if the bearings are mechanically imperfect so that an abrupt motion results with violent splashing of the contents of the shaking vessel.

Let us consider what happens in a so-called heterogeneous reaction of oxygen with a compound in solution. The reaction may proceed at the interphase between gas and liquid, or the oxygen may be dissolved in the solution and react with the compound in a homogeneous phase. The dissolved oxygen will, of course, soon be used up. Replenishment by dissolution of oxygen may be fast or it may be the slowest phase of the sequence of events. If the solution rate is slow, the observed rates will not be characteristic of the chemical system. Such falsifications have quite frequently crept into the literature. In order to avoid them, we take care that the oxygen is fed fast enough into the solution to keep it saturated with oxygen, that is, the rate of dissolution of the oxygen must be equal to, or higher than, the rate at which it is used up by the reaction. Obviously, the solution rate of the oxygen increases as the liquid-gas interphase is increased. An increase of the interface is obtained by vigorous shaking of the reaction vessel, and we check our experimental conditions by a series of experiments in which the shaking rate is increased from run to run (1, 2). The rate of the oxygen absorption increases with the shaking rate until the oxygen is dissolved as fast as it is used up. Then, the rate at which the oxygen recedes in the burette reaches a value which is characteristic of the chemical reaction in the solution. Higher shaking rates do not change this value because saturation of the solution is maintained.

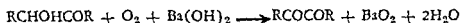
These considerations apply if the reaction occurs in the body of the solution and not in the interphase. If the reaction occurred in the interface as a truly heterogeneous reaction, the rate of absorption of oxygen would continue to increase with the shaking rate. The measurements which I am going to discuss were all made at shaking rates which were higher than necessary to keep the solutions saturated with oxygen.

Benzoin is a typical α -hydroxy ketone. Some α -hydroxy ketones

are physiological intermediates, and we have verified that the results obtained with benzoin apply to other aromatic α -ketols and also to the aliphatic α -ketol butyrolin (3). The end products in the autoxidation of the compounds are the corresponding acids.



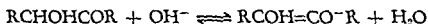
The reaction is catalyzed by alkali. If barium hydroxide is used as the alkali, barium peroxide precipitates and the supernatant solution contains the respective 1,2-ketone.



If the peroxide is not removed, it reacts rapidly with the diketone rather than with the α -ketol (1).



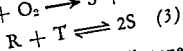
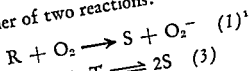
The kinetics of the autoxidation of benzoin are surprisingly simple. The rate is first-order with respect to the substrate and with respect to the hydroxyl-ion concentration. It is independent of the oxygen concentration. This shows that we actually measure not the reaction of benzoin and oxygen but rather a preceding reaction between benzoin and hydroxyl ion which is followed by a rapid reaction with the oxygen. The preceding reaction is the enolization of the benzoin.



The rate of enolization can be determined directly by measuring the rate of racemization of an optically active benzoin under nitrogen (4,5). It was found to be identical with the rate of autoxidation in oxygen. This identity of the two rates shows that the enolized benzoin is consumed by oxygen in a very fast reaction, before it can revert to the keto form. It is noteworthy that the rate of enolization, as measured by the autoxidation rate or by the rate of oxidation with Fehlings solution (6), is proportional to the electrolytic dissociation constants of the enediols and a function of the dissociation constants of the corresponding acids (7).

If the oxygen is not supplied as rapidly as it is consumed, a purple radical, similar to a semiquinone, is formed. It disappears upon abundant supply of oxygen. The purple intermediate may

be formed by either of two reactions:

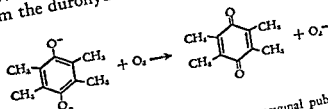


where R = the α -ketol, T = the diketone, and S = the radical.

In order to get behind the screen of enolization reactions, we must examine compounds which, from the very beginning, are present in the enol form. Well-known compounds of this kind are ascorbic acid and catechol, at least in one of the extreme structures of the resonance hybrid. Likewise, hydroquinone can be considered as the vinologue of an enolized α -ketol.

The autoxidation of hydroquinone, to begin with this compound, is quite important in photography. One of the chief reasons why sulfite is added to photographic developers is to avoid this wasteful aerial oxidation. The literature on the subject is rather confused. This is not surprising. If hydroquinone reacts with oxygen in a manner similar to benzoin, then we should get as primary oxidation products quinone and hydrogen peroxide, two compounds which, in alkaline solution, rapidly form hydroxyquinone. Moreover, quinone readily disproportionates in alkaline solution to form hydroquinone and hydroxyquinone. All these reaction products, in turn, react rapidly with oxygen. Moreover, the autoxidation of hydroquinone to quinone is in itself a complex reaction.

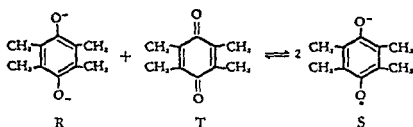
In order to eliminate the side reactions just mentioned, the reactive nuclear hydrogen atoms in hydroquinone are blocked by using tetramethylhydroquinone, durohydroquinone (8). This compound is autoxidized in a clean reaction to form duroquinone and hydrogen peroxide. The rate of reaction is proportional to the square of the hydroxyl ion concentration; that means that the reactive ionic species is the doubly charged durohydroquinone anion. The overall reaction could be written as a shift of two electrons from the durohydroquinone ion to the oxygen.



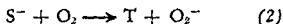
¹In order to avoid confusion, the equation numbers of the original publications are used for reactions and equilibria. One symbol (R, S, T, O_2^- , etc.) only, is used for the neutral molecule and any ionic species in instantaneous equilibrium with it.

However, the actual mechanism is not so simple. The reaction starts slowly and takes an autocatalytic course. Further experiments showed that the catalyst is the final reaction product, duroquinone. There is a slow reaction at zero quinone concentration, and a second reaction whose rate is proportional to the quinone concentration.

The rate of the catalyzed reaction is independent of the oxygen pressure. Again, we measure a reaction which precedes the reaction with the oxygen. In this case, it is the reaction of the durohydroquinone anion with the duroquinone, forming a semiquinone.



which then rapidly reacts with the oxygen.



This interpretation implies that the semiquinone equilibrium is not attained with immeasurably high speed, in the case of the tetramethyl compounds.

If we extrapolate our data towards zero quinone concentration, or if we add to the reaction mixture, before admitting oxygen, compounds which react with the quinone, we find a slow reaction of the durohydroquinone anion which does not appear to be catalyzed by the quinone. The rate of this reaction is proportional to the oxygen pressure.

The knowledge of the quinone catalysis and of the formation of the rapidly oxidizing semiquinone helped greatly in disentangling the reactions of the other compounds which we shall now discuss.

We proceeded by leaving out one methyl group after the other from durohydroquinone, that is to say, we studied the autoxidation of pseudo-cumohydroquinone, (trimethylhydroquinone) of the three xylohydroquinones, of toluhydroquinone and, eventually, of hydroquinone itself (9). In all these cases, the primary oxidation product is the quinone, while the oxygen is reduced to hydrogen peroxide. With hydroquinone, we had to use

a special reagent in order to catch the quinone before it reacted with the hydrogen peroxide; quaternized 2-methylbenzothiazole was added to the reaction mixture, and the quinone was identified spectroscopically as the dye, [(3-methyl-2(3)-benzothiazolyldene)-methyl]-*p*-benzoquinone (9). In all cases, the autoxidation rates are closely proportional to the square of the hydroxyl-ion concentrations and again the divalent ion is the most reactive ionic species.

In view of the results which had been obtained upon the addition of duroquinone to solutions of durohydroquinone, we added the corresponding quinones in varying amounts to the hydroquinone homologues. No effect was noticed when quinone was added to hydroquinone and to toluhydroquinone. A small catalytic effect showed up when *p*-xyloquinone was added to *p*-xylohydroquinone. However, addition of pseudo-cumoquinone to pseudo-cumohydroquinone causes a considerable increase in the autoxidation rate until about one-half mole of cumoquinone has been added; further addition of cumoquinone has no catalytic effect, i.e., the catalytic effect of cumoquinone reaches saturation (9, 10). The rate of the quinone-catalyzed reaction is independent of the oxygen pressure, but this independence ceases as the quinone catalysis reaches saturation, and in the presence of more than one-half mole of quinone, the autoxidation rate of cumohydroquinone is proportional to the oxygen pressure.

The autoxidation rate of toluhydroquinone and of hydroquinone is always proportional to the oxygen pressure. The question arises whether these reactions are not at all catalyzed by quinone, or whether we are measuring quinone-catalyzed reactions in a region where the catalysis has reached saturation. By analogy with the results with durohydroquinone and cumohydroquinone, respectively, the rate will be proportional to the oxygen pressure in both cases.

It is most probable that the autoxidation of hydroquinone and of its lower homologues is catalyzed by traces of quinone, and that it is unaffected by the addition of quinone because the catalysis reaches saturation at very low quinone concentrations. If, to solutions of hydroquinone, we add compounds which react very rapidly and completely with the semiquinone or the quinone, like, for instance, sulfite, cysteine, thioglycolic acid or some of the reducing agents like stannite and hydrazine, or ascorbic acid, we depress considerably the aerial oxidation of hydroquinone and of its lower homologues, obviously because the concentration of the active semiquinone is depressed either directly or by reaction with

Alpha-Ketols, Eneidiols and Hydroquinones

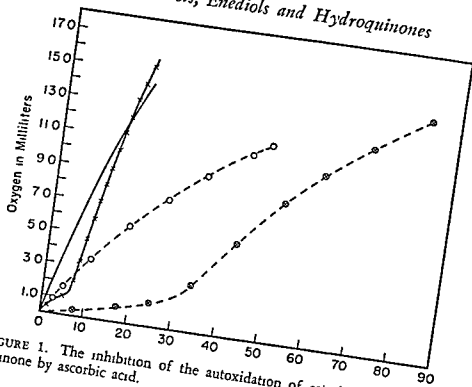


FIGURE 1. The inhibition of the autoxidation of catechol and hydroquinone by ascorbic acid.

— catechol
 —x—x— catechol + ascorbic acid
 - - o - - o - - hydroquinone
 e hydroquinone + ascorbic acid

the quinone in the equilibrium (11,12,13).

A similar effect is obtained if ascorbic acid and similar compounds are added to solutions of catechol, N-methyl-*p*-aminophenol, *p*-phenylenediamine, etc. (Fig. 1, 2). The inhibition persists as long as ascorbic acid is present, and usually the rates of autoxidation of the mixtures lie between the autoxidation rates of the individual components. This can be expected because the semiquinone of the catechol, the hydroquinone, the N-methyl-*p*-aminophenol, etc., is not completely removed by the added reducing agent. Moreover, the rapidly oxidized semiquinone of the ascorbic acid is formed in the reaction with the semiquinoid or quinoid autoxidation product. There are cases where this latter reaction even causes an acceleration of the oxygen absorption, as, for instance, when ascorbic acid is added to an autoxidizing solution of pseudo-cumohydroquinone, Fig. 3.

The stoichiometric nature of the inhibition is demonstrated by the action of cysteine on hydroquinone, Fig. 4. The sulfhydryl compound reacts with the quinone (12) and, maybe, with the

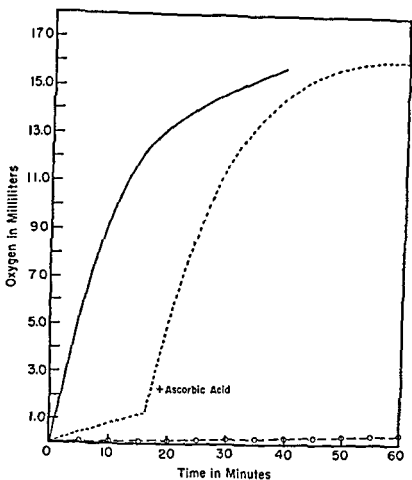


FIGURE 2. The inhibition of the autoxidation of N-methyl-p-aminophenol by ascorbic acid.

- N-methyl-p-aminophenol
- N-methyl-p-aminophenol + ascorbic acid
- o--o- ascorbic acid

semiquinone. Other sulfhydryl compounds have a similar effect. (11,12).

The oxygen absorption of a solution of hydroquinone which contains an excess of sodium sulfite, proceeds in four steps, viz., the autoxidation of the hydroquinone to quinone and hydroquinonesulfonate of the hydroquinonemonosulfonate to quinonemonosulfonate and hydroquinonedisulfonate of the hydroquinonedisulfonate to quinonedisulfonate, and eventually the autoxidation of the excess sulfite. The hydroquinonemonosulfonate and disulfonate, respectively, are formed by addition of sulfite to the quinones. Sulfite has a strong retarding effect on the autoxidation of hydroquinone, and most of the alkylhydroquinones, but it is

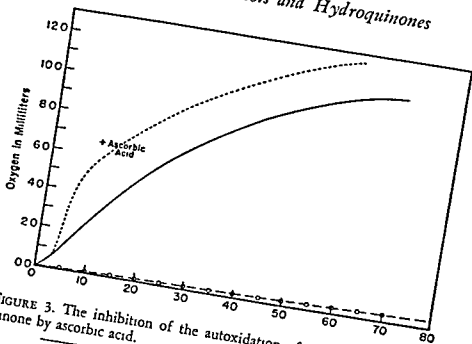


FIGURE 3. The inhibition of the autoxidation of pseudo-cumohydroquinone by ascorbic acid.
 — pseudo-cumohydroquinone
 pseudo-cumohydroquinone + ascorbic acid
 -o-o- ascorbic acid

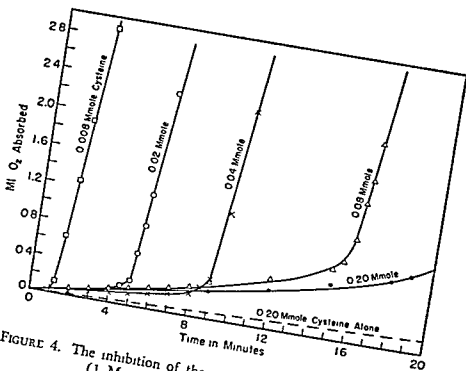
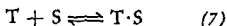


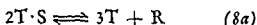
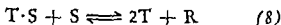
FIGURE 4. The inhibition of the autoxidation of hydroquinone (1 Mmole) by cysteine at pH 8.2.

without effect on the autoxidation of durohydroquinone. We believe that all these inhibitions are due to a removal of the semiquinone, either directly by reaction with the inhibitor or by the reaction of the inhibitor with the totally oxidized compound. They are less drastic than the inhibitions caused by a breaking of reaction chains.

The saturation of the quinone catalysis is not easy to understand. However, a rate dependency can be calculated which agrees with the experimental results if we assume that the reactive semiquinone is eliminated by the formation of a complex with the quinone.



and that this complex in interaction with a molecule of S or with another molecule of the same complex regenerates R and T (14).



These reactions in which semiquinone is "wasted" are favored by higher quinone concentration. Thus, with the addition or formation of more of the quinone, the concentration of the semiquinone reaches a limit. When this condition obtains, the catalysis reaches saturation, and the rate of oxygen absorption becomes proportional to the oxygen concentration.

Why is no saturation of the quinone catalysis reached with the tetramethyl compound, a saturation at a fairly high quinone concentration with the trimethyl derivative, a still lower saturation level with the dimethyl compound, and a level below the range of direct observation with toluhydroquinone and with hydroquinone? Duroquinone is stable in alkali even in the presence of peroxide and does not form complexes, pseudo-cumooquinone is still rather stable but forms complexes, and instability and the tendency to form complexes increase as we go down the ladder to unmethylated benzoquinone. The reactions which waste the semiquinone are favored as the protective methyl groups are left off, and the limiting concentrations of the quinone and of the semiquinone diminish accordingly. Thus, the kinetics of the quinone catalysis agree with the chemical nature of the compounds involved (15).

A particularly interesting α -ketol which is permanently enolized is ascorbic acid. It is a true aliphatic enediol. In hydroquinone, the two hydroxy groups are separated by a benzene nucleus

and the distance of about 6\AA renders them fairly independent of each other; if one hydroxyl is ionized, the charge is rather remote from the other hydroxyl group, and the second electrolytic dissociation constant is only about 100 times smaller than the first dissociation constant. While there are some indications of a very low reactivity of the monovalent hydroquinone ion with oxygen (9), this low reactivity is difficult to measure because of the small difference between the dissociation constants. With ascorbic acid (16), the two dissociation constants differ by a factor of more than 10^7 , and there are regions of pH in which we encounter almost exclusively the monovalent ion. It is well-known that heavy metals catalyze the autoxidation of ascorbic acid, and they were eliminated, therefore by the addition of a little cyanide and thiocyanate. With these precautions, we measured the rate pH dependence from pH 4.7 to pH 9.2 (Fig. 5). The slope of 1.0 at pH >7.2 shows a linear proportionality of the reaction rate to the hydroxyl-ion concentration. Inasmuch as at pH 7.2 practically all of the ascorbic acid is present as the monovalent ion, the slope of 1 show that the divalent ion is responsible for the oxygen absorption above pH 7.2. The reactivity of the divalent ion with oxygen at atmospheric pressure taken between pH 7.9 and 9.2 has the mean value of 4.9 min^{-1} . Below pH 7.2, the mean slope of the rate pH curve is 0.38.

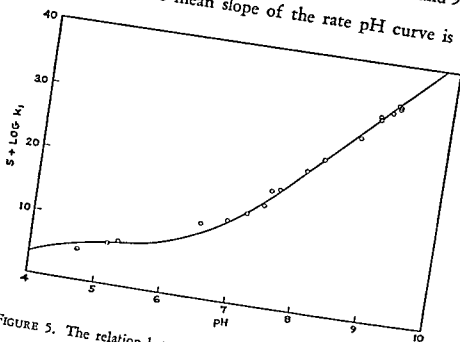
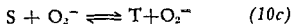
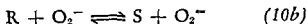
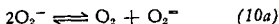


FIGURE 5. The relation between pH and the rate of autoxidation of ascorbic acid.

From this slope and the concentration of monovalent ion, the reactivity of the monovalent ascorbate ion was calculated and found to be of the order of $5 \times 10^{-3} \text{ min.}^{-1}$. The divalent ion reacts with oxygen 100,000 times faster than the monovalent ion. Even this low reactivity of the monovalent ion involves a quinone catalysis. In the region of high pH, the autoxidation rate is proportional to the oxygen pressure. However, the reaction of the monovalent ion is independent of the oxygen concentration. Again, the formation of a semiquinone by interaction of the ion with the oxidized ascorbic acid appears to be the rate-limiting step, and it is the quinone-catalyzed reaction which we observe with the monovalent ion of ascorbic acid.

I mentioned before that the autoxidation of ascorbic acid is also catalyzed by heavy metals. Copper is a particularly active catalyst. However, copper does not affect the reaction of the divalent ion, whereas it greatly accelerates the autoxidation of the monovalent ion of ascorbic acid (17). Again, the catalyst probably causes the formation of the highly reactive semiquinone of ascorbic acid. The power of this catalysis is illustrated by the fact that addition of four mg. of $\text{Cu}(\text{NO}_3)_2$ per liter increases the reaction rate of the monovalent ion by a factor of 10,000. The removal of heavy metals by cyanide and thiocyanate ion is a good example of inhibition by elimination of a catalyst.

It is not necessary in the present survey to discuss the effect of the formation of dimeric quinhydrone upon the kinetics of autoxidation (19). However, a word may be said about the fate of the perhydroxyl ion formed in reactions (1) and (2). It may be:



Reaction (10a) represents the spontaneous dismutation of perhydroxyl. Reactions (10b) and (10c) compete with this dismutation. The balance of the overall reaction will, in any case, be the same. If (10a) is much faster than (10b) and (10c), then reaction (10a) regenerates one-half mole of oxygen for each mole of oxygen consumed by reactions (1) and (2).

Reaction (10b) in combination with (2) would set up a chain mechanism. No signs of such a chain reaction were observed.

Reaction (10c) immediately following (1) in the same collision represents virtually a one-step bivalent oxidation. Such a

coincidence of (1) and (10c) would scarcely affect the two-step character of the overall reaction, because (1) is, in general, so slow that it is important only in the absence of T, i.e., in the very beginning of the reaction, and in the presence of inhibitors which remove T or S. However, from a theoretical point of view, such a two-step divalent oxidation would be interesting and I wonder whether there are any reasons why reaction (10c) should not follow (1) while the reactants are still caged by the surrounding solvent.

If reaction (10c) takes place as a separate step in reactions, the rates of which are limited by the formation of S, it will not affect the rate constants of the overall reactions. In reactions where the formation of S is rapid as compared with (2) and (10c), the reaction of O_2^- , according to (10c), may increase the rate of oxygen absorption and the rate constant. This increase is, of course, limited by the formation of O_2^- , according to (1) and (2), and by the dismutation, (10a).

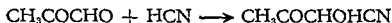
Our data, as far as they bear more directly on the topic of this conference, may be summarized as follows: The alkali-catalyzed autoxidation of enediols and hydroquinones is, at least in some cases, catalyzed by the fully oxidized compound T, by virtue of a reaction between R and T forming the highly reactive semiquinone S. Oxidations which are subject to this quinone catalysis are inhibited by a removal of T or of S. They might be considered as chain reactions where the final product acts as a chain link, and the mechanism of the inhibition might then be regarded as a breaking of reaction chains. On the other hand, the quinone may be considered as a common catalyst, and the inhibition as one due to the removal of a catalytic agent. Quinone catalysis may be classified as intermediate between catalytic processes in which chain reactions are started, and processes which are accelerated by virtue of a sequence of stoichiometric reactions in which the catalyst is regenerated. If, in biological processes, reaction chains were started by an enzyme and propagated by free radicals in solution, it would be difficult to understand the specificity of the respective reactions. Other mechanisms of catalysis and inhibition gain therefore in probability, and quinone catalysis may play a physiological role, provided that the appropriate enzyme systems exist.

this structure will be in resonance with



and some other electronic configurations which I need not write out. This resonance stabilizes the radical, lowers its energy content, and eases the oxidation of ascorbic acid in contrast to the conditions in glucose, e.g., where there are adjacent hydroxyl groups also, to be sure, but no double bonds. The concentration of the ascorbic acid semiquinone radical does not become high enough to make it directly recognizable, yet it will be high enough to exert its effects on oxidizability.

Weissberger: As far as I can see, it is the effect of the neighboring, negative CO group and, maybe, of the cyclic nature of ascorbic acid which render this compound so extremely autoxidizable. The effect of the negative group is in agreement with our results mentioned above. The catalytic effect of cyanide in the autoxidation of methylglyoxal is explained by the formation of an α -ketol that is activated by a negative group. The primary reaction is probably the formation of an α -ketol



which, due to the presence of the negative CN group, is rapidly enolized and oxidized. Meyerhof, *Bioch. Ztschr.* 159, 1432 (1925). Smythe, *Ber.* 65, 918 (1932).

Tobolsky: I should like to mention that a similarity exists between the maximum rates of autoxidation that we find and the maximum rates of oxidation found by Dr. Weissberger. A discussion of the maximum rate phenomenon in hydrocarbons will shortly be published in the *Journal of the American Chemical Society*.

I would also like to point out that during oxidation of hydrocarbons the products of reaction will often act as activators of hydroperoxide decomposition or as inhibitors of the propagation steps, and in certain cases the same substance can do both of these things.

Mansfield Clark: There is a problem which might be or might not be of interest to this group. It's the case of a compound which may be said facetiously to be its own antioxidant. By proper control of pH you can reduce neutral red and obtain either a compound that oxidizes readily or one that is stable in air.

I remember my surprise on taking the first out of an N₂-filled desiccator to see it oxidize explosively. The other compound is stable for years. We call it fluorescent X.

Michaelis: This reduction of neutral red to a fluorescent compound takes place also in cultures of *B. coli* and has been used by bacteriologists empirically as a test for *B. coli* in contrast to morphologically similar microorganisms.

Can the fluorescent compound be reconverted into the non-fluorescent one?

Mansfield Clark: As I recall, there are certain conditions under which you can regenerate the unstable compound from fluorescent X.

GENERAL PHARMACOLOGY AND TOXICOLOGY OF QUINONES AND HYDROQUINONES¹

JOSEPH SEIFTER

Weyeth Institute for Applied Biochemistry

THE USE of plants containing quinones, hydroquinone, and allied substances for the treatment of a great many diseases is as old as the practice of medicine. The current edition of the *United States Dispensatory* lists many of these still in use. The literature contains only a few studies of the pharmacological actions of the group or of individual members.

The chemical properties of quinones and hydroquinone are distinctive and rarely considered as ketonic or phenolic. The pharmacological properties, however, resemble those of phenols, partly because the latter give rise to Hq-Q-S² during the course of their metabolism.

Comparative toxicity. There is a considerable variation in the susceptibility of animals to intoxication by hydroquinones and quinones. The LD₅₀ of the vast majority in sensitive species lies between 50 and 300 mg./kg. of body weight. This figure covers such widely used compounds as Menadione and aloin. The oral toxicity is considerably reduced by the presence of colloidal substances such as proteins, which retard the rate of absorption. Aromatic diamines form a type of Hq-Q-S and have LD₅₀'s in the same range. Phenols, trinitrotoluene, acetanilid, salicylic acid, and other aromatic substances are up to a third as toxic.

Correlation of toxicity and chemical properties. No close correlation exists between the chemical properties of Hq-Q-S and the

¹ Individual details, referred to in the text, are omitted. How-
ever, the use of the term "quinone" is intended to include both quinone and hydroquinone, and is used in the same sense as in the literature.
² If a substance is used in the treatment of a disease, it is of course of use of

degradation, and on reoxidation to the quinone

toxicity. The probability of the correlation of either critical oxidation potential or inhibition of fat rancidity of amines, phenols, and quinones with their toxicity is more than suggestive (Table 1). Photographic reduction potential, effect on gasoline induction period, and rubber anti-aging cannot be correlated with toxicity. The statistical correlation of critical oxidation potential with the other chemical properties, however, is much better than with toxicity. Different methods of determining toxicity further complicate such studies. Hydroquinone by injection is only twice as toxic as phenol, whereas in aquarium studies, where local effects on the gills may predominate, it is one hundred times as toxic (Table 1).

Greater toxicity of the quinone form. Benzoquinone has greater oxidative effect than hydroquinone on nerve cells and produces clonic convulsions and fibrillary twitchings, whereas hydroquinone produces clonic twitchings characteristic of phenols. Hydroquinone is much more toxic *in vitro* for nerve and nerve endings under conditions of alkalinity and availability of oxygen, which favor the existence of the quinone form. Solutions of hydroquinone are toxic to isolated muscles only in the pH range of 6 to 9. Altering the metabolism and permeability of isolated muscle by a variety of agents did not yield results consistent with the greater toxicity of the quinone form.

Older animals are more resistant to hydroquinone intoxication than young ones, but can be made to be just as sensitive by increasing their oxidative activity. The quinone form, therefore, appears to be the more toxic, but enhancement of toxicity by the alarm reaction may occur since many of the procedures used to increase oxidative processes are also stressing stimuli.

Local effects on skin and mucous membranes. Local application of hydroquinones and quinones to the skin and mucous membranes results in pigmentation and various inflammatory changes. Sufficient corneal anesthesia occurs during exposure of the eyes to hydroquinone dust, that workmen may be unaware of such exposure until the onset of severe injury to the cornea and disturbance of vision. All layers of the cornea are penetrated and pigmented with yellow deposits of quinone. The effects on the mucous membranes may be seen also following systemic absorption and manifest themselves as diarrhea, salivation intense enough to cause hemoconcentration, and edema of the tongue, lips, and conjunctivae, which may result in marked distortion of the face. In this respect, the intoxication resembles that produced by p-phenylenediamine.

TABLE 1

Correlation of Oxidation Potential
(E_c) and Aquarium Goldfish Toxicity (FT)¹

Substance	E_c (volts)	FT (ppm)	RANK:		
			E_c	FT	Difference $E_c - FT$
P-Methylaminophenol (Metol, Elon)	0.603	0.5 (X·2)	1	2	-1
Pyrogallol	0.609	18. (X·1.8)	2	8	-6
Hydroquinone	0.631	0.287 (X·1.15)	3	1	+2
p-Aminophenol	0.673	2 (X·2)	4	3½	-½
p-Phenylenediamine	0.710	5.74 (X·1.74)	5	6	+½
Pyrocatechol	0.742	14. (X·1.4)	6	7	-1
2-Mercaptobenzo- thiazole	0.785	2. (X·2)	7	3½	+3½
Phloroglucinol	0.799	630. (X·1.6)	8	13	-5
p-Phenylglycine	0.833	20. (X·2)	9	9	0
Hydroquinone mono- methyl ether	0.843	200. (X·2)	10	12	-2
Resorcinol	1.043	57. (X·1.7)	11	11	0
Phenol	1.089	29. (X·1.15)	12	10	+2
Aniline Hydrochloride	1.135	5.5 (X·1.8)	13	5	+8

¹ Courtesy of Torald Sollman, unpublished data.

Pigment metabolism. Hydroquinone and some of its aromatic ethers bleach dark skin on local application, probably by inhibiting pigment formation at the site, thus resembling the antioxidant action of ascorbic acid on isolated skin. This form of toxicity was seen frequently among Negro workers engaged in compounding rubber with such agents.³

Suppression of pigment metabolism occurs also following prolonged systemic administration of hydroquinone and constitutes the mildest manifestation of chronic intoxication. Black cats gradually become gray and the depigmentation, like that of aging, involves in sequence whiskers, hair about the eyes, face, and head. Finally, the whole hide takes on a salt and pepper effect. Thirty mg. of hydroquinone per kg. of body weight administered three times weekly for six to eight weeks transforms black cats into fully gray animals which appear to be ill in no other way and, a few weeks after administration of hydroquinone is stopped, regain their original black hide. Black guinea pigs are depigmented to a lesser extent by the daily administration of benzylhydroquinone ether and some of its homologues.³ In cats there is a total inhibition of melanin formation, and in guinea pigs there is simply a reduction of melanin. Catechol does not have depigmentation properties.

The mechanism of the depigmentation is unknown. Certain indo-phenol dyes suppress the normal development of melanin in pigmented amphibia by action on the pituitary gland and by inhibition of tyrosinase. Physiological graying depends on disturbance of the enzymatic processes converting dihydroxyphenylalanine to melanin, and reduced pigment formation results from the disturbance of nutrition if the hair roots. The depigmentation by hydroquinone is analogous to that by ascorbic acid in that a co-enzyme system that can alter the physiological course of enzymatic pigment formation is interpolated. However, shaving, local application of cold, and other physical stimuli produce changes in the coat pigment of adult rabbits, and prolonged feeding of drugs producing an alarm reaction causes gray stippling in black dogs.³ The effects on pigment metabolism are due in part, therefore, to changes in the adrenals.

Effects on metabolism. Hydroquinones and diamines potentiate respiratory coenzymes. They are converted to the quinoid form by the tissue oxidases and are then capable of accepting hydrogen from substances undergoing oxidation. Hydroquinone increases

³ J Seifter, unpublished data.

the oxidation of ethyl alcohol and glucose *in vivo*. The hypoglycemia which may follow the ingestion of hydroquinone has been explained by this mechanism and was considered as confirmation of the use in folk medicine of hydroquinone-bearing plants in the treatment of diabetes. Hydroquinones, in common with other alarm stimuli, may also produce hyperglycemia, depending on the stage of alarm reaction when the blood samples are taken. They are, therefore, of no benefit in the treatment of diabetes.

Effects on the nervous system. The effects on the nervous system are manifested first by considerable restlessness and motor activity as a result of heightened activity in the spinal cord. Next, with involvement of the higher brain centers the animals make purposeless, uncontrolled movements and display erythismus. Twitchings of various muscle groups including those of the ears and eyelids, tremor and choreiform movements, and hypersensitivity to touch develop and are followed by anxiety and fright, malicious and aggressive behavior, mania, ataxia, and tonic and clonic convulsions. The muscle movements subside but can be provoked by stimulation. Finally, there is apathy, stupor, and paralysis as a result of depression of both the central nervous system and peripheral nerves. Death is due to respiratory and circulatory failure. Administration of large doses by vein results in prompt narcosis without the appearance of the other stages.

Resorcinol, which does not have a quinoid form, also produces convulsions with central and peripheral components. Both resorcinol and hydroquinone produce tonic convulsions in frogs with intact nerve connections between the muscle and the spinal cord, but clonic convulsions in frogs with severed nerve connections. Muscles which twitch rhythmically when immersed in solutions of hydroquinone or resorcinol undergo contracture in solutions of benzoquinone. These and the other findings cited indicate that the peripheral effects of hydroquinone are phenolic. Preliminary curarization of the muscle abolishes the twitchings, and excess of calcium suppresses them.

Effects on the blood. Methemoglobin formation and the asphyxia dependent upon it do not contribute significantly to the course of intoxication by Hq-Q compounds. The methemoglobinemia and the methemoglobinuria observed in such cases occur only post mortem, as test tube artefacts, or during terminal circulatory stasis, where there is opportunity for small amounts of hydroquinone to participate in the catalytic cycle involving oxidation to quinone and subsequent regeneration of hydroquinone

at the expense of conversion of hemoglobin to methemoglobin. The hydroquinones and hydroquinone monosulfuric acids present in blood and urine following the administration of quinones do not result from such catalysis during life, but arise by reduction and conjugation at some site other than the blood stream. The catalysis is very efficient, and if it occurred during life, doses of Hq-Q compounds known to be innocuous would produce intense methemoglobinemia. The mechanism which protects against this prevents the conversion of hydroquinones to quinones. This mechanism is not specific for Hq-Q compounds but protects hemoglobin also against arsines, stibines, bismuthines, and other substances activated by oxidation.

Subacute poisoning by Hq-Q compounds appears after the repeated administration of doses somewhat less than acutely toxic ones, or, as in the human cases described, several days following the ingestion of a single acutely toxic dose. The characteristic findings are jaundice, anemia, hemoglobinuria, and cachexia. The first sign is increasing fragility of red cells in sodium chloride solution. After a definite latent period during which a natural detoxification mechanism becomes exhausted, hemolysis sets in. This mechanism may involve the oxidation of glutathiol of the blood to glutathione by the Hq-Q-S. This same mechanism would account for the failure of methemoglobin to occur during life when glutathiol reserves are still available. An analogous protection by SH compounds occurs against the enzyme inhibitory action of diamines having stable semiquinone intermediates. The hemolysis together with depression of the bone marrow result in severe anemia. In a human case of fatal poisoning from the ingestion of 100 mg. of catechol per kg. of body weight, icterus was present on the fourth day and the red count was 1.3 million. The blood count of animals on the fourth day of poisoning is about 40% and the hemoglobin about 20% the initial value.

Progressive exhaustion of the bone marrow resulting in anemia, leucopenia, and thrombocytopenia is the most serious manifestation of chronic poisoning by Hq-Q compounds. Even compounds of the vitamin K series, excepting vitamin K₁, have this effect. Pthiocol, 2-methyl-1,4-naphthoquinone, and Menadione bisulfite in appropriate doses drop the red count and hemoglobin level. Quinones arising during the course of metabolism of aromatic drugs have been held responsible for granulocytopenia by depleting the bone marrow of glutathione. The anemia is not accompanied by hemolysis. The erythrocyte count in chronic poisoning descends slowly and reaches a level of stabilization from which a

further drop can be brought about by increasing the dose. The leucocyte count may decrease to less than 100 per mm³. Usually the bone marrow blockade is not so complete and reactivation is attempted, creating a picture characterized by reticulocytosis and leucocytosis.

Habituation. Habituation to Hq-Q compounds usually consists of acquiring tolerance to the actions of a fixed dose, but addiction and dependence on the pharmacological effects of one of these may occur as illustrated by troops resorting to powdered nutmeg when marihuana was not obtainable. Cats recently recovered from subacute hydroquinone poisoning develop passing signs of stimulation and excitement when administration of the drug is resumed, but do not develop any other signs of acute or chronic poisoning. Continued administration leads only to cachexia and death. Tolerance may also occur during the first course of administration of the drug. Continued administration during intoxication may also lead to tolerance with remission of the blood dyscrasia and nervous phenomena. With ideal dosing, blood dyscrasia and nervous phenomena do not occur at all, so that the first sign of intoxication may be the terminal cachexia. The tolerance extends also to the amount of hydroquinone needed to produce a lethal outcome. Doses which kill untrained animals in a few days are tolerated by trained animals for several weeks. Rodents do not have a high initial sensitivity to hydroquinone and, unlike carnivora, become less resistant during prolonged administration.

Whether tolerance or intolerance develops is important since these compounds have been suggested as antioxidants in foods, as anti-hyaluronidases in the treatment of rheumatic fever, and as anti-hypertensives. Hydroquinone itself is too toxic to be used as an antioxidant for foods or as a medicament.

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DISCUSSION

Ames: Rigor mortis follows immediately on the death of an animal killed with hydroquinone. This phenomenon is striking, for in a matter of minutes after an animal has expired, the rat is "stiff as a board." This is not true of many substituted hydroquinones with which a fatal dose results in a completely relaxed condition and rigor mortis appears following the usual time interval. The question can be raised whether hydroquinone is more easily oxidized than some of its substituted derivatives and thus affects the muscular system more rapidly.

RECENT DEVELOPMENTS IN THE CHEMISTRY AND METABOLISM OF VITAMIN E¹

STANLEY R. AMES and PHILIP L. HARRIS

Research Laboratories, Distillation Products, Inc.

THE MANNER in which vitamin E functions in the body has not yet been determined. Our present working hypothesis is that vitamin E acts primarily and specifically through some enzyme system and, secondarily, in a *non-specific manner as a physiological antioxidant*. The purpose of this discussion is to summarize, relative to this hypothesis, recent data concerning the chemistry and metabolism of vitamin E and to outline current trends in tocopherol-enzyme research.

A description of the isolation, proof of structure, synthesis and analysis of α -, β -, and γ -tocopherols is available in several excellent reviews (1,2,3). Recently, Stern, Robeson, Weisler and Baxter (4) discovered a new naturally-occurring vitamin E in soybean oil as the result of observing that the tocopherols of this oil gave anomalous results with the conventional Emmerie and Engel reagent. They isolated the new tocopherol and proved it to be 8-methyl tocol (d, δ -tocopherol). It is with particular reference to δ -tocopherol and the impact of its discovery in improving analytical methods that the chemistry of the vitamins E will be discussed.

CHEMISTRY

The four tocopherols (Fig. 1) are quite similar in chemical reactivity which leads to considerable difficulty in methods of individual estimation. Mild oxidation with gold chloride converts the tocopherols to their respective *p*-quinones (Fig. 1), possessing an absorption maximum at about 260 m μ . These *p*-quinones can be reduced with sodium hydrosulfite to the corresponding hydro-

¹ Communication No. 116.

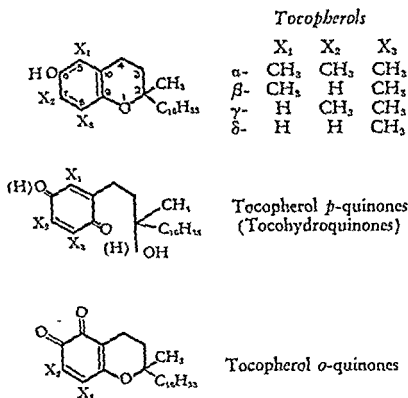


FIGURE 1. Tocopherols and their Quinones.

quinones and the latter can be recycled with sulfuric acid to yield the original tocopherols. These reactions are the basis of a number of analytical procedures.

Oxidation with ferric chloride likewise leads to the formation of *p*-quinones. The ferrous iron is usually determined by the photometric estimation of the red-complex formed with α, α' -dipyridyl according to the widely used method of Emmerie and Engel (5). δ -Tocopherol is unique in that, with the ferric chloride-dipyridyl reagent, it gives a slow, steady increase in color intensity after the initial rapid oxidation is completed. Color development is about the same on a molecular basis for the four tocopherols when a reaction time of 2½ minutes is chosen. At 10 minutes, however, δ -tocopherol gives approximately 22% greater intensity than the others. This increase in color intensity probably indicates that δ -tocopherol is oxidized beyond the *p*-quinone stage (6). A procedure for the assay of total tocopherols in mixtures containing δ -tocopherol has been developed (6), using a standardized time for the development of the color.

More vigorous oxidation of α -, β -, and γ -tocopherols with silver nitrate leads to the formation of the red *o*-quinones (Fig. 1) which with minor differences in absorption spectra have an absorption peak in the range 460-480 $m\mu$. The product formed from δ -tocopherol has a broad absorption maximum at approximately 435 $m\mu$ (4) which suggests a mixture of reaction products.

Vigorous oxidation of tocopherols with nitric acid likewise yields red pigments of *o*-quinones. This reaction, discovered by Furter and Meyer (7), has been widely used as the basis of photometric assay procedures. Under the conditions of this test a distinctly different compound is formed from δ -tocopherol from the other tocopherols. It has an absorption maximum at 373 $m\mu$ (4) and since the quinones from the other tocopherols have absorption maxima at around 460-480, an analytical method could be developed for δ -tocopherol by determining the intensity of absorption at the specific wave length of the δ -tocopherol pigment.

The tocopherols can be broken down into a number of characteristic products by oxidative degradation with chromic acid. For instance, from α -tocopherol a number of compounds were isolated which included dimethyl maleic anhydride, diacetyl acetone, a C_{21} lactone, a C_{18} ketone and a C_{16} acid. From the determination of the constitution of these oxidative breakdown products the structure of the tocopherols themselves have been established.

The various tocopherols differ in their resistance to atmospheric oxidation, as shown by Stern *et al.* (4). Olive oil solutions of each of the tocopherols were exposed to air at 55°C. in an oven and the percentage recoveries at various time intervals were determined by the ferric chloride-dipyridyl method. The stability of the tocopherols to atmospheric oxidation decreases in the order δ -, γ -, β -, and α -tocopherol. This order is the same for their antioxidant activity and the reverse of their biological potency.

It seemed from an examination of the structural formulae of the four tocopherols that it would be possible for β -, γ -, and δ -tocopherol to couple with diazonium salts. However, it was found that while γ - and δ -tocopherols coupled readily with diazotized *p*-nitroaniline, β -tocopherol did not (8,9). Diazotized *o*-dianisidine was found to be a more stable and desirable reagent for this purpose. A differential method for the determination of mixtures of α -, γ -, and δ -tocopherols has been worked out by Weisler *et al.* (9). It depends on the relative difference in color intensity of the coupled γ -, and δ -tocopherols in sodium carbonate and in potassium hydroxide solutions. By measuring the color intensity of each solution at two wave lengths, it is possible to calculate the amount

of γ - and the amount of δ - in mixtures of tocopherols. Recently, a method has been developed by Quaife (10) for the quantitative determination of the individual tocopherols in mixtures. It is based on the reaction of the non- α -tocopherols with nitrous acid as described by Scudi and Buhs (11). The yellow nitroso derivatives are fractionated by chromatographic absorption and eluted separately for spectrophotometric analysis. The concentration of α -tocopherol is then determined by subtracting the total of the β -, γ - and δ -tocopherols, determined separately, from the total tocopherols determined by the Emmerie and Engel procedure. This method has been applied to vegetable oils following preliminary concentration of the tocopherols by molecular distillation.

The tocopherols have a free hydroxyl group and can be esterified by any of the conventional procedures. A number of esters have been prepared, all of which involve the phenolic hydroxyl in position 6 of the tocol nucleus. The more common of these are the acetates, palmitates and alphanates, and the water-soluble phosphates and the slightly water-soluble succinates. In general, these tocopherol esters are somewhat more effective biologically than the original tocopherols probably because of a greater stability against oxidative destruction. Tocopheryl phosphates have assumed the greatest prominence because of their water solubility and resemblance to other vitamin phosphates of biological importance.

METABOLISM

The metabolism of the tocopherols will be discussed under the following divisions: absorption and excretion, catabolism, transport, deposition, and physiological function. Reviews by Hickman and Harris (12) and Mason (13) have covered the general aspects of the metabolism of vitamin E, but there are still many gaps in our knowledge of this process.

Absorption. Vitamin E fed in normal quantities is apparently readily absorbed. The plasma tocopherol levels in rats (14), dogs and humans rise as the result of the oral administration of α -tocopherol. Oral administration to rats of either tocopheryl phosphate or tocopheryl acetate, as shown by Engel (14), also increases serum tocopherols, indicating rather rapid hydrolysis of the esters. However, the amount of tocopherol appearing in the serum following the administration of *dl*, α -tocopheryl phosphate was only about one-half that observed when an equal quantity of *dl*, α -tocopherol was fed. Other reports (15), however, have shown that in

a human the ingestion of d, α -tocopherol and d, α -tocopheryl phosphate induced comparable "tolerance" curves. Another factor involved in the absorption of vitamin E which should be emphasized is bile salts. Lack of bile salts in the intestinal tract reduces the absorption of tocopherols, as shown in rats (16) and dogs (17) with bile fistula. Since this might lead to an induced deficiency of vitamin E which in turn would have a detrimental effect on liver function, a vicious cycle would ensue.

Excretion. Studies by Cuthbertson, *et al.* (18) indicate that while only 3-15% of a daily dose of 3.5 mg. of tocopherols appeared in the feces of rats as much as 25% of large doses was excreted. Hines and Mattill (19) also reported that "large amounts" appeared in the feces on the administration of massive doses. The fecal excretion of the tocopherols is roughly proportional to intake, but apparently nearly complete absorption occurs during normal intake. A similar generalization can be made for urinary excretion. Cuthbertson, *et al.* (18) found no tocopherol in the urine of rats on normal diets, but on a diet high in vitamin E, they found 19-day excretions of 0.6-0.8 mg. of material spectrophotometrically similar to tocopherol. However, other investigators could not demonstrate vitamin E in the urine of rats receiving 100 mg. of tocopherols per day (19) or in cow's urine.

Catabolism. The majority of absorbed tocopherols probably undergoes degradation since only a small amount is excreted in the urine. This is not surprising since vitamin E is one of the more labile substances in the body. The occurrence of tocoquinone *in vivo* was reported by Scudi and Buhs (11) who reported 3 parts of tocoquinone to 1 part of tocopherol in dog plasma. However, Hines and Mattill (19) could not find tocoquinone in liver, muscle, or urine.

Most of the vitamin E can be extracted from tissues with alcohol or an alcohol-skellysolve B mixture but there appears to be some residual, tocopherol-like material which is tenaciously held by the tissue. Extraction of muscle tissue with acidified alcohol is reported (19,20) to remove an additional 20-50% of material reacting chemically like tocopherol. However, in the case of beef muscle (20) or beef liver (21), extraction with acidified alcohol failed to remove additional reducing substances. The constitution of this tightly bound tocopherol-like material has not yet been determined, but it has been variously suggested as being a protein conjugate or a phosphoric acid ester of tocopherol.

It has been implied (12) that the non- α -tocopherols are converted *in vivo* to the α - form. The relative biopotency of the β -,

γ -, and δ -homologues compared to α -tocopherol may be dependent on the ability of the test animal to methylate them to the α - form. However, if γ -tocopherol is fed to humans, all the increase in plasma vitamin E appears as γ -tocopherol (15). Preliminary work also indicates that when γ -tocopherol is fed to laying hens, γ -tocopherol is secreted in the egg (22) and when the feeding is continued γ -tocopherol almost completely replaces the α -form in the yolk.

Transport. The normal concentration of tocopherol in blood plasma ranges from 0.9 to 1.2 mgs.%, although variations from 0.1 to 3.0 mgs.% have been observed. Treatment of plasma with ether or hydrocarbon solvents alone slowly extracts the tocopherols, but prior or simultaneous addition of ethanol, rapidly extracts the vitamin (23). These observations suggest the existence of a protein-tocopherol conjugate (24). It has been found *in vitro* that relatively large quantities of α -tocopherol can be dissolved in plasma by either of two procedures (23). One method consists in slowly adding a concentrated solution of tocopherol in dioxane to the plasma, with rapid stirring. The resulting solution is somewhat turbid but may be clarified by high-speed centrifugation. A preferable procedure is to homogenize a mixture of plasma and α -tocopherol in a Potter-Elvehjem glass homogenizer followed by centrifugation. An α -tocopherol concentration of 200-300 mgs.% in plasma can be readily achieved in a stable, slightly opalescent solution. The plasma proteins were fractionated with either ammonium sulfate or ethanol at low temperature and all the resulting fractions contained α -tocopherol. A large number of amino acids, partially-hydrolyzed proteins, and native proteins have been tested for their ability to solubilize α -tocopherol, and only native proteins have been found satisfactory. Some of the proteins were lipid-free so it appears that vitamin E transport in the body may involve a tocopherol-protein conjugate without the mediation of lipids.

Storage. An excellent summary of the ability of the rat to store vitamin E under conditions of normal and of abnormally high intakes has been given by Mason (25). The highest normal concentrations of vitamin E were found in spleen, mammary gland, lung, and heart. When enormous doses of α -tocopherol were administered, large quantities were found in the liver and increased amounts in muscle and body fat. Recently Quaife and Dju (21) analyzed human tissues and found the largest quantity of vitamin E in body fat. A 70 kg. male was estimated to contain about 3.5 g. of vitamin E of which over one-half was in the body fat. On

the basis of keeping time, Lundberg *et al.* (26) found the maximum deposition of α -tocopherol in fat depots of rats occurred 7-10 days after feeding a single dose. The non- α -tocopherols were either less completely absorbed or more slowly utilized.

Physiological Function. The four tocopherols are considerably different both in biopotency and in their antioxidant activity. On the basis of rat bioassay, d, β -tocopherol is 40%, d, γ -tocopherol is 4%, and d, δ -tocopherol is 1% as potent as natural α -tocopherol. A recent investigation (27) of the relative biopotency of natural and synthetic α -tocopherols and their esters showed that d, α -tocopherol and its esters are 136% as potent as the corresponding dl-forms; also, α -tocopheryl succinate and vitamin E esters in general are 162% as potent as α -tocopherol in the free form. It is interesting to note that the relative activities of α -, β -, γ -, and δ -tocopherols as antioxidants for vitamin A acetate in olive oil at 39°C. were 100, 130, 180 and 270% respectively (4).

A discussion of the metabolism of vitamin E would be incomplete without some reference to the probable function of tocopherols in enzymatic mechanisms. Investigations in this field fall logically into three main divisions. First, are studies of the changes in enzyme systems which result from a vitamin E deficiency; second, and very closely allied, are studies of changes in enzyme systems in which the tocopherols have been added *in vivo* and *in vitro*, and, third, are studies dealing with the quite separate consideration of the action of the tocopheryl esters *in vivo* and on isolated enzyme systems.

Severe vitamin E deficiency leads to a form of muscular dystrophy in many animals characterized pathologically by the gradual replacement of normal muscle fibres with fibrous tissue. Dystrophic tissues have an increased oxygen consumption (28) which results in a high rate of respiration for the whole animal. A deficiency of vitamin E is unique in that it results in a stimulation of respiratory mechanisms. In addition, the dystrophy syndrome is accompanied by alterations in chemical composition and in functional behavior, of which the most striking is decreased muscle creatine and marked creatinuria. The succinic dehydrogenase system is apparently unaffected (29), but changes have been noted in the response of the lactic dehydrogenase system to digitoxin (30). General disturbances in phosphorylation mechanisms have been noted in dystrophic muscle, including the depression of coupled phosphorylations of creatine and of adenosine triphosphate (31). The decrease in cholinesterase content of the tissues in vita-

min E deficiency implies a close association of vitamin E and acetylcholine synthesis (32).

The administration of α -tocopherol results in an immediate decrease or even complete remission of the various changes associated with muscular dystrophy. When administered to normal animals, it has been reported to modify the metabolism of lipids and phospholipids (33), to enhance phosphorylations (34), and to improve the metabolism of carbohydrates (35). The addition of α -tocopherol, even when solubilized, to *in vitro* systems has in most cases no effect. However, α -tocopherol in minute concentrations has been shown to stimulate acetylcholine synthesis (36). Furthermore, it inhibits lipoxidase (37), probably due to its action as an antioxidant.

The study of the action of the tocopheryl esters on a number of isolated enzyme systems is currently of great interest. α -Tocopheryl phosphate (α -TPh), because of its water solubility, has played a commanding role. Orally administered α -TPh can be readily hydrolyzed (14) in the body and many of its *in vivo* reactions parallel those resulting from the administration of unesterified tocopherols. α -TPh, added *in vitro*, has been shown to markedly inhibit practically every enzyme on which it has been tested. The inhibition of the succinic oxidase system apparently involves both a specific action and a non-specific secondary mechanism involving calcium removal and subsequent inhibition by oxalacetate (38). Diphosphopyridinenucleotidase is likewise strongly inhibited by α -TPh (39). α -TPh has been reported to have no effect on coupled phosphorylations (31) but to stimulate phosphocreatine synthesis under certain conditions (40). α -TPh inhibits a number of other enzymes including liver acid phosphatase (41), fatty acid oxidase (42), protease, trypsin and is antithrombic (43).

Since α -TPh inhibits such a varied group of enzymes, it seems to be functioning as a non-specific protein inhibitor (23) and, in fact, its structure resembles that of an anionic detergent such as dodecyl sulfate. α -TPh probably forms a complex with any native protein or enzyme and effectively blocks the active centers. Considerable confusion exists with respect to the comparison of the actions of α -tocopherol and its phosphate. The latter is not known to exist in the body and active hydrolytic mechanisms are probably adequate to release free tocopherol. α -TPh is not an antioxidant, possesses no oxidation-reduction potential and is oxidized with difficulty (explaining its relative ineffectiveness as a sparing agent in the intestinal tract). Frequently direct compari-

sons are made between *in vitro* concentrations of α -TPh and *in vivo* levels of tocopherol. Such comparisons are generally unwarranted. The *in vitro* effects of α -tocopheryl phosphate probably bear no relationship to the biological functions of vitamin E.

Much work has been done in the past with dystrophic tissues, following the classical approach of relating vitamin deficiency to lowered enzyme activity. However, such a study is greatly complicated, since it is impossible to distinguish between primary and secondary effects. Further investigations of this type would suffer from similar difficulties. Studies currently in vogue relative to the action of α -tocopheryl phosphate on isolated enzyme systems while relatively easy to perform are difficult to interpret in terms of *in vivo* metabolism. This difficulty will remain until α -TPh is isolated from tissues or proved otherwise to exist physiologically. Suitable techniques for the addition of free tocopherol to isolated enzyme systems have recently been devised and should be relatively easy to interpret. It would appear that in the future the greatest promise of success in establishing the physiological function of vitamin E lies in this type of investigation.

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DISCUSSION

Mason: In comparison with the biological temperatures at which the tocopherols act, the temperature of 55°C. at which these antioxidant studies were carried out is high. Are there studies at more biological temperatures?

Ames: The relative antioxidant activity of α -, β -, and γ -tocopherols was reported by Hove and Hove (J. Biol. Chem. 156, 623 (1944)) to be dependent on temperature. At physiological temperatures they reported that the antioxidant activities of the three tocopherols were about equal and that α -tocopherol had the greatest antioxidant potency followed in order by β - and γ -tocopherols. However, Stern, *et al.* (J. Am. Chem. Soc. 69, 869 (1947)) found that at 39°C, δ -tocopherol was the best antioxidant for both vitamin A and carotene, the others in the order of decreasing effectiveness being γ -, β -, and α -tocopherol.

Quackenbush: We have some data on ethyllinolate in which we used carotene as an indicator of the stability, in which we found the reverse of what we got on glycerides. Although we have not investigated that very extensively, we might expect to get differences in the order of stability depending upon the substrate.

Ames: At what temperature is that?

Quackenbush: 37 degrees centigrade.

Ames: Naturally the temperature is an important consideration in all determinations of effectiveness of various substances as antioxidants. It is difficult to make valid comparisons between the results of 70-100°C experiments and data obtained at physiological temperatures or *in vivo*. The reaction products may be entirely different and even the mechanisms can be altered with such changes in temperature.

I would like to raise for discussion the relationships between the different products of tocopherols obtained by different oxidizing agents. Under mild conditions, the *p*-quinones are formed but with stronger oxidizing agents such as nitric acid, the *o*-quinones are obtained. Can the *o*-quinone be formed in a step-wise reaction with the *p*-quinone as an intermediate or are two different mechanisms involved in the formation of the *o*- or *p*-quinones?

Michaelis: Did you ever try to make the hydroquinones of these orthoquinones?

Ames: I do not know of any data.

Weissberger: The reduction of quinone with hydrazine might

be of use. In the case of benzoquinone the reaction is very fast and the yield almost theoretical. Besides the hydroquinone, only nitrogen seems to be formed.

György: What happens when different tocopherols are given? Alpha tocopherol is absorbed. What about the others?

Lundberg: When individual tocopherols are fed in equal amounts to rats that are low in vitamin E, α - and β -tocopherols are deposited in the adipose tissues to a greater extent than is γ -tocopherol.

Hickman: May I give you Dr. Baxter's theory on that? You will find that vegetable nature operating as it must in the cold at various temperatures and with different oxidation requirements has to have a selection of tocopherols with different potentials. On the other hand, the animal body which operates at a standard temperature has a preferred oxidation potential which is met best by α -tocopherol. If this is the status of the tocopherol in the vegetable kingdom, I suspect that with improved analytical methods probably all the possible homologues and isomers of tocopherol will be discovered before we are done. Of the three common tocopherols, gamma and delta act as the most powerful antioxidants through the intestinal tract and perform a valuable service there, but the only one that is absorbed in any substantial quantity and arrives at its final destination in tissue is α -tocopherol. Now you might ask what is the clinical significance of the various tocopherols. At the present time, the tendency is for the manufacturers to convert the tocopherols into an ester because they will survive better in the intestinal tract and be better absorbed. However, in point of view of human needs, it would seem that what is required is a nice blend of all the tocopherols plus α -tocopherol esters, so that the α -tocopherol can reach its destination in high yield while the others perform their extra services in the intestinal tract.

György: You are implying that only α -tocopherol can be absorbed from the intestinal tract.

Hickman: I would guess that α -tocopherol has a unique position.

Mackenzie: I would like to know the services which are performed in the intestinal tract?

Hickman: Quackenbush and coworkers showed the sparing action on carotene. Since then we have found that we can pick up the sparing action of tocopherols on many other oxidizable substances in the intestinal tract that we have convenient means of measuring.

One of the important things which we would like to know is—

what does changing the tocopherol content of the intestinal tract do to intestinal flora? Sometime ago my associates and I tried to interest a group to examine what happened to the polio virus in the intestinal tract when the tocopherol intake was varied. They did a few spot experiments and found that when enough tocopherol was present, there was 100% survival of the virus in the stools, which we considered to be a very important observation. Thousands of chemotherapeutic substances have been examined without finding any correlation whatsoever with polio, but here at least is a positive correlation even if it is in the wrong direction. Because the response was in the wrong direction, the experiments were stopped by the Polio group who failed to realize that it presented a magnificent means of starting an investigation, for if an intestinal antioxidant failed to reduce polio virus in the intestinal tract, presumably a prooxidant operating in the other way would be of great value. In conclusion I would say that the whole question of what the antioxidants do in the intestinal tract, including their influence on the intestinal flora, is wide open for examination.

Michaelis: Do I understand you correctly that the phosphate ester in the intestines acts as an antioxidant?

Ames: No, tocopherol phosphate cannot function as an antioxidant.

Lebninger: What is the correspondence between the various tocopherols in the antisterility test and in muscular dystrophy?

Ames: The four tocopherols have approximately the same relative activities in relieving experimental muscular dystrophy in rabbits as in preventing fetal resorption in rats.

Lebninger: How can you modify the tocopherol molecule and still have an active compound?

Ames: It is possible to substitute an amine group for the hydroxyl in position 6 of α -tocopherol and have an equally active compound in the antisterility test. If the side chain is modified in any way, the compound is not active biologically.

Fieser: The amine probably is readily transformed into a hydroxyl group in the body.

Hickman: The point that interests me is that you must not alter the tail on the tocopherol and yet you can replace the hydroxyl with an NH_2 group.

OBSERVATIONS ON THE BIOLOGICAL EFFECT OF TOCOPHEROL IN LIVING ORGANISMS

PAUL GYÖRGY

Nutritional Service, University of Pennsylvania School of Medicine

TOCOPHEROL, as well as ascorbic acid, may be considered as typical "antioxygenic" substances, representing in food products and in the living organism true analogues of technical antioxidants. It is known that in inert systems their role is comparable to that of ordinary, technical antioxidants. However, their function and the mechanism of their action in the living organism are still obscure. In particular, our knowledge regarding the participation of tocopherol in biological reactions is very limited. The symptomatology of tocopherol deficiency (infertility, muscular dystrophy, vascular and nervous disturbances) offers no definite indication or explanation for the mode of its action. At our previous conferences, allusion was repeatedly made to the observations of the Mackenzies, and McCollum, Mason, and Dam on the interaction of tocopherol and unsaturated fatty acids in living animals. These are at least consistent with the assumption that tocopherol may act in the living body through retardation of oxidation involving unsaturated fatty acids. It is hoped that through further study of tocopherol-action in biological systems, more light may be shed on the underlying physico-chemical reactions.

The following presentation includes observations on (a) the production and prevention of dietary hepatic necrosis, and (b) the occurrence of hemolysis and hemoglobinuria in rats following administration of alloxan and related substances. This work has been carried out in collaboration with Miss Catharine S. Rose, and in part in collaboration with Dr. Harry Goldblatt.

TOCOPHEROL AND DIETARY HEPATIC NECROSIS

Acute necrosis (massive or zonal) is one of the specific forms of hepatic injury which may be reproduced by purely dietary

means (1,2). Until recently, the opinion was widely held that acute dietary necrosis is the result of a specific deficiency characterized by lack of the sulfur-containing amino acids, cystine, or its precursor, methionine. In rats kept on a semi-synthetic diet low in casein, acute fatal necrosis, or its sequela in surviving rats, i.e., *post-necrotic scarring*, is encountered in 40-50% of the experimental animals, with the rest showing at the end of the experimental period (120 - 150 days) signs of *more or less marked diffuse fibrosis (cirrhosis)*. With the addition of choline to the diet cirrhosis may be completely prevented, but the incidence of necrosis is increased slightly. The irregular occurrence of massive necrosis in rats fed a diet low in casein was claimed (2) to be due to the quality of the casein which even when given in small amounts supplied methionine (and cystine) not too far below the prophylactically effective level.

By substituting yeast for casein the intake of sulfur-containing amino acids may be further lowered. Thus, it seemed to fulfill expectations, when reports appeared (2,3), that in rats fed rations with yeast as the sole source of protein, massive necrosis became a regular occurrence. According to these observations, the development of hepatic necrosis was again completely suppressed by prophylactic supplements of cystine or methionine.

In spite of the seemingly conclusive evidence, as offered by these yeast experiments, objections may be raised against the identification of dietetic massive necrosis as simple cystine deficiency. For instance, attempts to produce massive hepatic necrosis were unsuccessful, in several laboratories, with yeast rations (4), or with any other diet forms (5). These negative results were recorded in spite of the fact that in all the rations used the intake of sulfur-containing amino acids was kept at a very low level and apparently within the same limit as in the yeast experiments with their high incidence of massive hepatic necrosis. Substitution of vegetable fat for lard in a diet low in casein resulted in the almost complete disappearance of acute zonal or massive hepatic necrosis. Addition of tocopherol to rations containing lard also suppressed the development of acute hepatic necrosis (6). The inclusion of tocopherol in the experimental diet may also explain the failure (7) to produce hepatic necrosis in rats kept on diets such as yeast rations, which without supplements of tocopherol were successfully used for the production of hepatic necrosis.

Thus, *tocopherol* emerges as an important additional protective dietetic factor in the etiology of hepatic necrosis. With regard to the development of massive hepatic necrosis, *tocopherol* may com-

pensate for the absence of cystine and/or methionine and *vice versa*. In exceptional instances the mutual effectiveness of cystine (methionine) and tocopherol is not applicable. In this connection mention should be made of recent observations (8) on hepatic injury in rats, which was presumably of the necrotic variety, that was found to be preventable by tocopherol, but neither by cystine nor by methionine (8).

The interchangeability of sulfur-containing amino acids (cystine, methionine) and vitamin E as leading etiological factors, makes it difficult to accept a pure deficiency state as the basis of massive hepatic necrosis. The question arises whether both cystine and vitamin E act more through a detoxifying mechanism, perhaps involving retardation of chemical (oxidative?) reactions than through substitution of missing or exhausted intermediary building stones. The fact that hepatic necrosis may develop in a very short time, sometimes only in a few weeks after the experimental animal was put on the basal yeast diet (3), speaks more in favor of a toxic factor than of pure deficiency. In this connection observations made in our laboratory with the use of various types of yeast as constituents of the experimental rations, may be of interest.

Fleischmann's Pure Dry Brewer's Yeast, Type 50B represented the sole source of protein in one group of experiments. In a second set of experiments a British Baker's yeast was employed, obtained through the kindness of Professor H. P. Himsworth, London. Fermentation tests showed no activity for the American yeast, whereas the British yeast exhibited slight but definite activity. The analytical data for N and total S were:

	N	S
	%	%
American Yeast (Fleischmann's Type 50B)	7.83	0.86
British Yeast (United Yeast Co. Ltd., London)	6.76	0.65

The first group of animals was kept on the following ration: American yeast 16, cornstarch 30, lard 50, salt mixture 3, cod liver oil 1; supplemented daily with a solution of thiamine, riboflavin, pyridoxine and pantothenic acid. In the sub-group of 12 rats fed the above basal diet, acute necrosis of the liver developed in 6 animals. Thirteen control rats receiving supplements of tocopherol to the basal diet remained free from necrosis throughout

the whole period of observation (up to 150 days). Initial average weight in both groups was found to be 92 and 96 Gm., with a final average weight of 56 and 59 Gm. respectively. Daily food intake was low in both groups, in the average 3.4 and 3.2 Gm. respectively. If yeast with its low content in sulfur-containing amino acids was selected as a source of protein to promote the development of cystine deficiency, the low food intake should have certainly accelerated it. Further, lard and cod liver oil with their high content of unsaturated fatty acids should have increased the requirement for vitamin E. In spite of these experimental conditions all seemingly favorable for the production of acute hepatic necrosis, the latter developed in only half of the experimental animals kept on the basal diet. By using a similar high-fat ration, but with another type of yeast in the diet, Himsworth and Glynn (9) observed massive hepatic necrosis in practically all their experimental animals.

In order to elucidate this discrepancy, experiments were set up in which Fleischmann's Pure Dry Brewer's Yeast was compared with the active British Baker's yeast with regard to their ability to promote the production of dietary hepatic necrosis.

At the suggestion of Professor Himsworth, the following basal diet was used: yeast 18, cornstarch 79, salt mixture 3. The experimental animals were offered daily 8 Gm. of this mixture, to which 0.5 ml. of arachis oil and 2 drops of cod liver oil were added just before feeding. In addition, the rats received the usual B vitamins, in form of a combined aqueous solution (1 ml. daily).

Out of 14 rats fed the above ration, with the British yeast as the sole source of protein, 13 animals died between the 100th and 144th experimental day from acute, hemorrhagic necrosis. Its massive character, extending over many lobules and even lobes, distinguished it from the usual more zonal form of necrosis (central and midzonal) seen in rats fed rations low in casein. In all macroscopic and microscopic details the findings conform to those given by Himsworth for his animals kept under identical conditions. Only *one* rat of the group of 14 animals survived the experimental period of 200 days without any observable pathological change of the liver. It is the first time that in our laboratory massive necrosis was observed as a truly regular occurrence. In two sub-groups of animals, consisting of 7 rats in each, and fed the same basal diet (with the British yeast), supplements of tocopherol (3 mg. daily) or cystine (50 mg. daily) prevented the development of hepatic necrosis throughout the 200 day experimental period.

In substituting American yeast for the British yeast, without any further change in the experimental conditions, hepatic necrosis was successfully prevented, even in a group of 14 rats which received *no* supplements of cystine or tocopherol. The rats of this group were sacrificed after 200 days in the experiment; their livers were found perfectly normal, without any sign of acute or healed necrosis. In 2 groups of 7 rats each, fed the same basal diet (containing the American brand of yeast), but exposed to cold (4 hours daily in a cold room) or to muscular exercise (2 hours daily in a rotating cage) respectively, hepatic necrosis remained conspicuously absent. All these experiments were carried out concurrently with rats of the same strain, age, and identical dietary history.

The complete absence of hepatic necrosis in the groups of rats kept on the experimental ration with the American brand of yeast as source of protein, in contrast to the *regular* occurrence of exceptionally severe, massive, hemorrhagic necrosis, when British yeast replaced American yeast in the ration, requires further elucidation. The greater analytical value for total S in the American yeast may indicate a higher content of cystine (or methionine) than that present in the British yeast. Furthermore, it is conceivable that the British yeast, being an active yeast, is less well digested and utilized than the inactive American yeast (10). On the other hand, the average food intake in a group of rats fed the diet with the American or British yeast was nearly equal (in the average 6.5 Gm. daily in the first 14 weeks for the group with the American yeast, and 6.1 Gm. daily in the first 14 weeks for the group with the British yeast). Even when all these various factors are considered, the difference in sulfur-intake in the two groups does not appear to be distinct enough to give a satisfactory explanation for the *complete* absence of hepatic necrosis in one group and for its *regular* occurrence in the other, and further, it is difficult to see how and why tocopherol should compensate for insufficient supply of cystine or methionine.

This brings us back to the question as to whether the interchangeability of cystine (methionine) and tocopherol may primarily be due rather to an underlying detoxifying mechanism than to the restitution of a deficiency condition. If this were the case, then the regular occurrence of massive hemorrhagic hepatic necrosis seen in rats on a special yeast ration might be traced either to the presence of a toxic factor or to the absence or low content of a special detoxifying constituent in this yeast. This detoxifying factor cannot be vitamin E which appears to be absent in all types

of yeast (11), and it is probably only partly represented by sulfur-containing compounds. Its identification requires further studies.

Thus, the phenomenon of massive hepatic necrosis provides an impressive illustration of the difficulty encountered in distinguishing between mechanisms based primarily on deficiency or intoxication. These mechanisms may be, as for instance in the case of dietary hepatic necrosis, thoroughly interwoven, and do not permit a distinct separation of factors relative to deficiency or to intoxication.

As detoxifying agents, vitamin E and cystine (cysteine?) will probably act through the intermediary of some oxidation-reduction reactions, although the possibility of different points of attack resulting in the same effect, i.e., in the prevention of hepatic necrosis, cannot be excluded. Be that as it may, dietary acute hepatic necrosis offers a good example of the biological role of vitamin E.

HEMOLYSIS FOLLOWING ADMINISTRATION OF ALLOXAN AND ITS PREVENTION BY TOCOPHEROL

According to recent studies of Houssay and Martinez (12), rations low in protein or high in lard reduced, while supplements of methionine increased the resistance of rats to alloxan. Houssay and Martinez judged the beneficial or deleterious effect of a given ration by the number of rats which survived one week after the administration of alloxan. Substitution of butter or olive oil for lard or supplements of choline were without any appreciable effect. Oleomargarine and corn oil were slightly beneficial. It was previously known (13) that cystine (cysteine) when given just prior to alloxan may also counteract the toxic effect of alloxan.

The experiments of Houssay and Martinez contain no specific information on the possible role of tocopherol in the prevention of alloxan intoxication. As a matter of fact, the rations used by them seemed to be rather low in tocopherol, and, in consequence, a deficiency of tocopherol might have complicated the experimental findings. It is of further interest that the grouping of the noxious and beneficial dietary agents in the experiments of Houssay and Martinez is reminiscent of, and almost analogous with, that which governs to a considerable extent the production of massive hepatic necrosis.

The possibility that tocopherol might exert the same beneficial protective effect on necrotic changes in the pancreas (liver, kidneys) following the administration of alloxan, as it does in the

prevention of massive dietary hepatic necrosis in rats, made a special study of its role in alloxan intoxication desirable.

Female rats of the Sprague-Dawley strain weighing between 90-120 grams were divided into several groups and fed different rations for a period of one month. At the end of this preparatory period and after all-night fasting the rats were injected intraperitoneally with 160 mg./kg. of alloxan, given in the form of a freshly prepared solution.

A large variety of semi-synthetic dietary rations was used. In the first set of experiments the diet was low in protein (casein 10%) and high in fat (40%), supplemented with the usual salt mixture and B vitamins, the rest being made up by sucrose. Fat was given either in the form of lard, or of vegetable shortening, or of coconut oil, in all instances 38%, with cod liver oil (2%) added. Special groups received supplements of choline (25 mg. daily) or of methionine (50 mg. daily) or of tocopherol (3 mg. daily). In later experiments the proportion of casein was raised to 20%, and for a few groups of rats to 40%. In several sets yeast was also added (5%) to the various rations. Finally a low fat diet was also introduced. In these groups fat was completely excluded from the basal ration and was represented only in form of small supplements of percomorph oil (3 drops once a week) and of corn oil (3 drops daily).

Rats often died within the first two days after the administration of alloxan, and several of these, as well as a fair proportion of the surviving animals showed signs of more or less intensive hemoglobinuria. Hemoglobinuria was accompanied by intravascular hemolysis and a rapid fall in the red blood cell count, occurring almost immediately, with a peak a few hours after injection of alloxan. Hemoglobinuria was *never* observed in rats fed rations rich in tocopherol, either in the form of tocopherol-supplements or of vegetable shortening with a high content of tocopherol. On the other hand, a varying but often high proportion of the experimental rats kept on rations free from, or very low in, tocopherol showed hemoglobinuria after the intraperitoneal injection of alloxan. Supplements of methionine or high-protein, high-lard diet were, in the absence of tocopherol, without any appreciable effect on hemoglobinuria. Addition of yeast to the ration was followed in most instances by a reduction of hemoglobinuria. In addition to alloxan, its reduced forms (alloxantin and especially dialuric acid) have also produced hemolysis when injected into rats fed a tocopherol-free diet. In contrast, alloxanic acid has proved to be inactive. In view of the well-known affinity of allophanic acid

to tocopherol and its possible relation to alloxan, it should be noted that the injection of allophanates produced no hemolysis under circumstances in which injection of alloxan was followed by hemolysis and hemoglobinuria. Administration of alloxantin and dialuric acid to rats fed rations containing tocopherol or supplemented with tocopherol was never followed by hemolysis.

Ninhydrin, a close chemical relative of alloxan, has an especially strong hemolyzing effect in animals fed a tocopherol-free diet. At the same time ninhydrin produces no diabetes.

The minimum hemolyzing doses for alloxan, dialuric acid and ninhydrin when given intraperitoneally, are as follows:

Alloxan: 160 mg./kg.

Dialuric acid: 40 mg./kg.

Ninhydrin: 10 - 25 mg./kg.

Even when given in excessively large doses (500 mg./kg.) ninhydrin will not produce hemolysis in rats fed rations containing tocopherol.

It is of special interest that hemoglobinuria (hemolysis) occurred independently from diabetes. The dietary factors, such as tocopherol or yeast, which suppressed or at least reduced hemoglobinuria, had no influence on the incidence and intensity of diabetes. Thus, the pharmacological-toxicological action of alloxan seems to be based on two independent components, one being responsible for the hemolysis, the other for the diabetes.

These observations were made on about 500 rats fed the various semi-synthetic rations, half of which (fed to about 250 rats) were free from or very low in tocopherol. In rats kept on the regular stock diet, intraperitoneal administration of alloxan was not followed by intravascular hemolysis. Thus, some degree of tocopherol deficiency must be a prerequisite for the production of intravascular hemolysis under the influence of alloxan. In this connection, it is particularly noteworthy that rats previously fed the regular stock diet, require only 7 to 10 days on a tocopherol-free diet before the sensitivity of the red blood cell to alloxan develops. This is the more surprising when one considers the fact that rats of similar age and weight have to be kept on the tocopherol-free diet for months before they may be considered as depleted of their tocopherol-stock and ready for bioassay test. In contrast to rats fed the regular stock ration, animals primed for several weeks with large daily doses of tocopherol (3 mg.) showed prolonged resistance to the hemolyzing effect of alloxan.

There are indications that the *in vivo* hemolysis by alloxan may also be demonstrated and studied *in vitro*. This will offer the opportunity of analyzing the underlying reaction. The chemical nature of alloxan as a triketone apt to form semiquinones, and that of tocopherol as a typical antioxidant, make it probable that their interaction may involve radicals and chain-reactions, similar to reactions occurring in inanimate systems. Therefore, the assumption is perhaps permissible that this special biological system will offer an example for a biological reaction to be considered as true "antioxidation".

Finally, a possible clinical implication of the observation here reported may be mentioned. The question whether alloxan or similar compounds may play a part in the pathogenesis of human diabetes, has remained so far unanswered. Alloxan is too unstable under the conditions of biological systems to be traced, isolated and identified in and from the living organism. On the other hand, it is logical to postulate that impairment of insulin production, which in the final analysis initiates the diabetic reaction, must be due to some other determining cause or factor, perhaps of the type of alloxan. In this connection the following circumstantial evidence for the existence of alloxan or similar compounds among the metabolites of a diabetic or even pre-diabetic person might deserve some consideration.

It is known that newborn infants of diabetic or pre-diabetic mothers are characterized, in addition to other pathological manifestations, by the almost regular occurrence of erythroblastosis and extra-medullary hematopoiesis, to a large extent similar to that seen in so-called Rh-incompatibility (14). No explanation has yet been offered for this obvious sign of intrauterine destruction of red blood cells (hemolysis?). It is a generally accepted fact (15) that the transfer of vitamin E via the placenta does not occur very efficiently, and, in consequence, nursing rats may be easily depleted of their small vitamin E stores. Thus, we may further conclude, or at least assume, that alloxan or alloxan-like substances circulating in the maternal blood, and also appearing in the fetal circulation, will not produce hemolysis of the maternal erythrocytes due to normal tocopherol stocks of the mother, but will hemolyze fetal red blood cells, owing to the "physiological" vitamin E deficiency of the fetus. Obviously, this hypothesis requires further study and examination.

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DISCUSSION

Michaelis: This is the opportunity to discuss the behavior of alloxantin. Alloxan can be reduced, by a bivalent reduction, to dialuric acid. There exists an intermediary reduction product, alloxantin, which is analogous to quinhydrone insofar as its molecular weight is double that of a free radical on the oxidation level between alloxan and dialuric acid. It may be considered as a molecular compound of the two, quite in analogy to quinhydrone. Alloxantin is colorless and can be prepared in pure form in the crystalline state. In solution, it dissociates to a very large extent into its components just as does quinhydrone. The analogy goes even farther: alloxantin, when dissolved in a strongly acid solvent to saturation, or almost to saturation, establishes an oxidation-reduction potential, as Bijlman has shown. In our own laboratory,

E. S. Hill and I showed that even in less acid solutions, pH 4 - 6, and at quite low concentration, alloxantin establishes a potential in the presence of an iron salt as catalyst, and under those conditions, dialuric acid yields a potentiometric-oxidative titration curve of such a slope as to indicate a bivalent reversible redox system without there being an intermediate, univalent step in any concentration great enough to be detectable with this method. This is, also, analogous to the oxidative titration curve of hydroquinone in acid solution. Now, just as in the case of hydroquinone, the existence of a semiquinone radical even in acid solution, although only to a minute extent, will not be denied, so, also, in the case of alloxantin, an analogous free radical will be postulated to exist, which on dimerization becomes the well-known alloxantin. As one searches for evidence of such a radical, one has to recall a well-known color reaction of alloxantin. On mixing its solution with barium-hydroxide, a violet amorphous precipitate is established. Concentrated NaOH reacts similarly, but the color fades out rapidly. It is impossible to obtain the barium compound in crystalline form. I have tried in vain to obtain experimental information about its magnetic properties. Nevertheless, there is good reason to assume that this is a barium salt of the free radical. This interesting case should be investigated more fully. Anyhow, the similarity in many respects of alloxan with a quinone is obvious.

György: To what extent does the free radical exist?

Michaelis: May I answer the question as follows. When quinone is reduced in an acid solution, the potentiometric titration curve has a slope indistinguishable from that of a bivalent redox system with no intermediate semiquinone step at all. However, the method is not very sensitive. The maximum amount of semiquinone, in equilibrium with the quinone and the hydroquinone, exists at 50% reduction. If this maximum amount is smaller than about 5% of the total concentration of the quinone, the method, as it is now, cannot distinguish it from 0%. However, there is a method to arrive at a fairly accurate estimate of such percentages or extrapolation, based on the slopes of redox potentials as depending on pH, according to the rules established by W. M. Clark. This principle can be applied as follows: in quinone, or, better, in duroquinone or phenanthrene quinone (which is less susceptible to irreversible deterioration in alkaline solutions), the semiquinone formation can be easily and precisely measured by potentiometric titration in alkaline solutions of pH 10 to 12. Applying the prin-

ciples just mentioned, the curves can be extrapolated into neutral and acid pH values. This extrapolation shows that the semiquinone formation never vanishes entirely, even in acid solutions. Although the maximum amounts obtainable under equilibrium conditions may be small, they are more than sufficient to account for the effects of those semiquinones on the kinetics of oxidation and for the reversibility of the system, even in acid solution.

Fieser: The action appears to be something like that expected of a quinone. It seems to me it would be worth-while to see if you could produce either the diabetic effect or the hemolytic effect, or both with suitable quinones, for example, with a fully substituted benzoquinone such as duroquinone. If such a substance produces one effect or the other, or both, the observation might be suggestive of an ultimate interpretation. Also, it seems to me that experiments utilizing lard are subject to uncertainty. Lard is a very complex and variable mixture of fats, and more exact information might result from the use of a pure, synthetic fat. Yeast, also, is a complex mixture. Is ergosterol the effective component? Can the effect be reproduced by the use of pure ergosterol? Saponins produce hemolysis at very low concentrations, and the hemolytic effect is counteracted by cholesterol or ergosterol. Possibly some similar complex molecule is involved here. The only point I want to make is that from the chemist's point of view, it seems desirable to narrow the problems down by finding out what components are actually involved.

György: Dr. Fieser is certainly right in drawing your attention to the fact that lard is a very poorly defined chemical compound. His criticism in this respect applies, also, to other constituents of semi-synthetic rations generally used in dietary experiments, such as proteins (casein), yeast, cod liver oil, starch, etc. On the other hand, the fact remains that neither we nor animals eat pure chemical compounds. Nutritional research has to draw its conclusions from experiments in which protein, fat, yeast, etc. are used and the only caution we are generally following is not to lose sight of the fact that contaminants or deterioration of the food constituent may falsify our results. With regard to our present experiments, they were of a comparative nature and the comparisons were made between the several experimental groups, themselves. Inasmuch as we have used the same batch of casein, lard, vegetable shortening, cod liver oil and yeast throughout all the experiments, conclusions for comparison may justly be drawn.

The beneficial effect of yeast on hemolysis caused by alloxan was much inferior to that exerted by tocopherol.

Rusch: In connection with Dr. György's report, it is of interest that we have found that L-cystine fed at a level of 0.5% inhibited the formation of hepatic tumors in rats due to 4-dimethylaminoazobenzene. Inhibition with methionine was slight but no effect was noted with tocopherol.

In a recent issue of Science (108:308, 1948), Lazarow and his associates demonstrated that cysteine and glutathione combined with alloxan. They suggested that the protective effect of these agents against alloxan diabetes was the result of a reaction of the alloxan with the -SH groups. Will glyoxal, also, react with glutathione?

Hickman: I feel that the great importance of György's papers is that they present a wealth of new experimental facts. The facts stand for themselves; it is with the interpretation that I take issue.

Surely, we are faced with two separate questions. One is specific and local and is concerned with the particular series of events that occur when alloxan is introduced into the body cavity of the rat. The other concerns the general question of the balance between prooxidants and antioxidants in the animal. We are permitted to discuss both aspects because Dr. György has introduced both.

The point has been raised, here, as to whether the physiologic antioxidants can operate through these semiquinones. A corollary is that the full quinones must be prooxidant. In any live organic material, there must be a balance between the prooxidants and the antioxidants, including all kinds of substances in both classes, whether naturally or artificially introduced. The antioxidant status of the animal body is, thus, a *buffered* one. The balance in any individual may set towards the oxidative side or the reductive side or be "normally" midway, whatever that is. The same is true with diet, which may be oxidative or reductive in quality, so we must ask ourselves nutritionally not so much "Does this diet contain a natural antioxidant, or so many milligrams of α -tocopherol or what-not?" but "Of the many antioxidants and many prooxidants in this diet, is there a balance appropriate to the animal consuming the diet?" At this stage of the argument, we do not have to inquire into the exact chemistry of an individual agent, such as tocosemiquinone on one side and linoleyl peroxide on the other. The important point is the balance. A well-known example of the balance is the production of dystrophy symptoms in the rabbit; either by withholding tocopherol or by giving cod liver oil. Applying this theme to Dr. György's paper on the two brands of yeast, it would suggest that the important point is to compare the over-all oxidative-reductive status of the two yeasts—is one

fresh and the other rancid?—rather than to search for a new “factor.” Specifically, is the British yeast prooxidative? I suspect that it is.

Now, we may discuss the alloxan experiments. I do not think we need worry whether alloxan can produce a semiquinone. All we need to ask ourselves is whether alloxan, its fragments or metabolites, is likely to serve as a physiologic prooxidant. With active ketol and potential quinone groups, it is inevitable that during the degradation of a large quantity of alloxan, much prooxidant must be produced. To introduce 160 milligrams into a little animal like the rat is almost as drastic as introducing the same quantity of gunpowder, touching it off, and then arguing whether the result is due to the physiological effect of sulfur dioxide!

I will assume, therefore, that one generalized action of alloxan is to force the oxidative balance of the body to the “pro” side, and this balance can be restored by adding sufficient antioxidant in the form of α -tocopherol.

Why is one fat soluble antioxidant physiologic and another not? Two qualities are required, availability to reach the ultimate site of operation in the tissues and the correct redox potential. Apparently, only α -tocopherol has both these in optimum degree. Many other substances have these qualities in lesser degree or balance, and they exert physiologic effect when introduced into the right place in the organism or given in sufficient amount.

One of the researches one would like to do in continuing Dr. György's experiments is to discover what other prooxidative substances will duplicate the action of alloxan, showing that we are dealing, indeed, with a general case. In the case of the yeasts, it would be interesting to see whether simultaneous administration of ascorbic and p-aminobenzoic acids would not neutralize the prooxidant effect. Again, would not some of the peroxidized fats that Dr. Swift has been talking about have the same action as the “rancid” yeast?

A final word, now, concerning alloxan and diabetes. Do we have to inquire into the intimate effect of alloxan on the cells of the pancreas? I think not. The whole body has been thrown on the prooxidant side, and the pancreatic cells cannot survive. If I were having the pleasure of continuing Dr. György's experiments, I would try a number of antioxidants equivalent to tocopherol and a number of prooxidants equivalent to alloxan, and find out whether they would not duplicate or neutralize one another in causing and curing these symptoms. I think we would

find we were dealing with a case of buffering between prooxidants and antioxidants, the same kind of buffering which we find in all of the physiologic processes.

Mackenzie: One question on which I would like to comment has been raised repeatedly at our first two Conferences, namely, "Do chain reactions occur in living systems?" It was asked yesterday and again today. It expresses the hope that something of what we have learned from Dr. Taylor, Dr. Mesrobian and Dr. Tobolsky concerning the action of antioxidants in stopping chain reactions *in vitro* may be extended to the understanding of the function and mode of action of biological antioxidants in living organisms. The question has not only expressed a hope, but also a doubt, for frequently it has taken the form, "Yes, but do chain reactions occur *in vivo*?"

Now the body is loaded with antioxidants like cysteine, glutathione, ergothionine, ascorbic acid, the tocopherols and so on. Each of these compounds is potentially capable of interrupting an oxidation chain. Moreover, there is a marked synergistic relationship in the effectiveness of antioxidants. Even phosphoric acid and its monobasic salt augment in a synergistic manner the antioxidant effect of the tocopherols. (Golumbic, Mattill; Transactions of the First Conference.) We would not ask Dr. Tobolsky for a striking demonstration of chain reactions in some system of his to which he had added all of the antioxidants he could lay his hands on. So it is with the living cell. It seems to me that the occurrence of chain reactions in the normal cell, chains of sufficient length to be readily detected, is improbable because of the antioxidants that are present.

However we are still confronted with the question as to whether or not living systems possess the mechanisms for starting chain reactions, that is to say whether chain reactions would occur in the absence of antioxidants. Certainly there are components of the normal diet that will autoxidize in the laboratory in an aqueous medium containing dissolved oxygen. Furthermore, the dietary components are transformed in the body by enzyme reactions into hundreds of intermediary metabolites, many of which are known to be relatively unstable. It seems probable that some of these compounds might undergo chain reactions in which dissolved molecular oxygen, ever present in the tissue fluids, plays a role. This problem is subject to experimental approach, both by studying the autoxidation of biological compounds in simple and complex systems *in vitro*, and by studying the chemical pathology of organisms deprived of antioxidants. A beginning has already been made.

When vitamin E is removed from the diet, the unsaturated fatty acids in the tissues undergo changes that are suggestive of *in vitro* autoxidation and polymerization. When this biological antioxidant is removed, oxygen consumption (of muscle tissue) is increased. The vitamin A stores of the liver disappear at a faster rate, just as A would disappear from an aqueous or colloidal system *in vitro* in the absence of antioxidants. There is a resemblance between the effects of a deficiency of this antioxidant in the test tube and in the body. It provides at least a tentative answer to the question, "Do chain reactions occur in the body?" The answer is, "They can occur, but their occurrence is normally prevented by biological antioxidants."

Swift: Dr. Mackenzie has mentioned the point that chain reactions would not be expected in the presence of such antioxidants as we have in our bodies. However, let us remember that tocopherols do not prevent, but inhibit gross oxidation *in vitro* and, possibly, *in vivo*. It has been shown that tocopherols efficiently exert an antioxidant effect at low levels of concentration. When the antioxidant effect of tocopherols was tested at progressively higher levels, it was found that the efficiency progressively diminished until, finally, levels were tested at which the addition of tocopherols decreased the stability of test fats. Furthermore, the rate of peroxide accumulation in fats stabilized by tocopherols was found to be inversely proportional to their tocopherol content. The rate of peroxide accumulation is quite rapid during the induction period of fats stabilized with 0.2% of tocopherols and quite low during the induction period of fats stabilized with 0.02% tocopherols.

Mackenzie: I was speaking of antioxidants limiting the length of oxidation chains so drastically as to make their demonstration in biological systems very difficult and the normal occurrence of long chains improbable in oxidative reactions.

Lundberg: What was the temperature at which you conducted your experiments?

Swift: From 60 to 100°C.

Hickman: Dr. Swift, isn't there something that you have left out, namely, that you get your maximum stability, not just with tocopherol, but, as Dr. Mackenzie pointed out, where you have small amounts of tocopherol, together with ascorbic acid? It is only when you use very large quantities of tocopherol without such adjuvants, that you get a completely new phenomena. I would assume that in the normal animal body, where everything

is working properly, nature must have learned to provide these adjuvants so that a complex, not a simple situation is provided.

Swift: The antioxidant system tocopherol-phosphatides has been studied. The antioxidant potency of tocopherol was increased by the addition of phosphatides, but, otherwise, the characteristics of the behavior of the tocopherol as antioxidant were apparently not changed.

Michaelis: If more peroxides accumulate during the induction period when tocopherol is increased, wouldn't that simply show that tocopherol does not act at the peroxide formation, but at some later stage?

Swift: One explanation I can think of is that peroxide formation is not entirely prevented by the antioxidant and that the peroxides which form are protected against decomposition, hence they accumulate.

Michaelis: How do you conduct your experiments?

Swift: By following the rate of peroxide accumulation at temperatures ranging from 60-100°C. We have been studying, also, peroxide decomposition. We have started with kilograms of oxidized fats or esters and finished with milligrams of oxidation products. However that may be, we have identified 2-undecenal as a product of the decomposition of methylhydroperoxide oleate and hexanal, 2-octenal and 2,4-decadienal as products of the oxidation of cottonseed oil. The latter three aldehydes we presume to be decomposition products of the hydroperoxides of linoleic acid.

In the stomachs of test animals, you may find variations in the ability of different species in eliminating such toxic substances as are produced during fat oxidation. This would explain, possibly, the differences in results of tests with, say, rabbits and chickens.

Tobolsky: Where do we get cleavage?

Swift: Bergstrom identified two hydroperoxides of methylloleate, in which the hydroperoxide group was located at the 9th and 13th carbons. In forming these peroxides, a shift of double bonds must occur. Cleavage of the 13-hydroperoxide could yield hexanal and cleavage of the 9-hydroperoxide could yield 2,4-decadienal. We have explained the isolation and identification of 2-octenal by assuming that the 11-hydroperoxide was present and, likewise, was subject to cleavage.

It is obvious that such aldehydes as 2-octenal and 2,4-decadienal are very reactive and that condensation, polymerization and other reactions could be expected. From these aldehydes could be formed such pigments as those mentioned by Dr. Mackenzie.

Tobolsky: Hydroperoxides are often decomposed by reaction products of oxidation. In certain cases, we have isolated hydroperoxides that are very stable. Even these will decompose very rapidly in the presence of a reducing agent.

Mason: The problems that you are faced with in these very interesting studies are, in part, those that have existed for many years in vitamin E research, namely, diets high in fat (lard and cod liver oil) intensifying symptoms or lesions which are minimal or, even, absent with low fat diets. Furthermore, when such high fat diets are, also low in choline as a result of low protein content, fatty infiltration and cirrhosis of the liver are added to the picture. Here, too, much as in your studies, tocopherol supplements have but little influence upon liver morphology, but do exert a remarkable effect upon the growth and general well-being of the rat.

One other point which concerns the alloxan diabetes question. There has recently been reported a fetal resorption in rats given alloxan which resembles that seen in vitamin E deficiency, except that it begins rather specifically on the 12th day of pregnancy and is associated with continued growth and maintenance of the placenta to term. It would be of interest to know whether this type of resorption is due to the action of break-down products of alloxan or whatever the mechanisms are that are responsible for the hemoglobinuria in your experiments, and, also, whether tocopherol would prevent this alloxan resorption.

Mattill: We must not lose sight of the fact that vitamin E is, itself, readily autoxidizable. Existence of an optimum concentration for stabilizing purposes has often been demonstrated *in vitro* as well as in the alimentary tract. When it is present in too high a concentration, its antioxygenic effect is overtaken, as it were, by its own autoxidation.

The varied responses to deprivation of vitamin E are amazing, but the literature on tissue respiration is full of such differences, depending on species, the age of the animal, or on minute variations in the conditions, such as the presence of unusual substances.

Thus far, our attempts to relate vitamin E to some of the known enzyme systems has been only indifferently successful. Our Dr. Hummel showed that in certain species, the phosphorylation of creatine is diminished in dystrophic muscle. The energy which should have gone into this process is wasted, even as the muscles, themselves, waste away. More recently, some other enzyme processes, concerned primarily with the metabolism of amino acids, have been found altered in dystrophic muscle, such as transamination, whereas other components or processes are quite unchanged

from those in normal tissue.

When the events in cellular metabolism are precisely known, the role of vitamin E in their regulation will also become evident, and the term "physiological antioxidant" will have a more exact meaning.

Hickman: I have written down on a piece of paper here "conditioning agent," "specificity," and "a screw-driver hypothesis." Those are three paragraphs taken from a general paper, which I wrote about six months ago.

A variety of situations condition nutrients in the field—the refrigerator, the cookpot, the intestinal tract and the interior of the body. Evidently, "*in vitro*" and "*in vivo*" are insufficiently descriptive, and we need a new term, which I will call "*in transitro*," to cover the period when the nutrient is passing from the gut to the circulation, and, thence, to the distributive centers in the body. All kinds of disasters can happen to essential chemicals, whether exogenous or endogenous, during this interim period.

Let us consider an analogy with the well-known physical process of distillation. In a distillation flask, there are millions of layers of molecules, all *waiting* to distill, but it is only the active *top* layer that actually does distill at any moment. If the others decompose before reaching the surface, we have no yield of product and the distillation is a failure.

So it is in the living organism. A few molecules at any moment are engaging in specific reactions, mostly at catalytic interfaces. The rest of the molecules, and at any instant, the overwhelming majority of them, are just in storage or transport, waiting their turn to react. All through time, all sorts of unwanted side reactions could destroy these materials before they discharge their essential physiologic mission.

A physiologic antioxidant is a substance that controls or prevents unwanted oxidations and side reactions. It is the vitally important police force that looks after the extracellular activities of the body's precious metabolites. As I see it, δ -tocopherol is the chief of the materials that look after these matters in the fatty phase of the body, just as ascorbic acid, p-aminobenzoic acid, and the phospholipids do in the watery parts and fat-water interfaces. Let me give you a striking example of prooxidant and antioxidant activity of the extracellular sort that goes on in the body, yet has no part in enzyme reaction. If you will feed large quantities of vitamin A to a chicken, it will lay an egg with a white yolk. The chicken's health and the egg are both unchanged; just the yolk is white. What has happened? Vitamin A is a specific

prooxidant for the carotenoids, and it has bleached these specifically without touching anything else in the system. If you give extra tocopherol to the chicken with the vitamin A, the eggs become yellow again because the E has buffered the A. These reactions have gone on in solution in the body just as if it were a test tube and no enzyme or functional chemistry has been involved. An important change has been promoted or stopped without its having anything to do with what we call the primary processes of living. The action has been, secondary, non-specific.

I want to give you two examples to illustrate specificity.

The top of a toothpaste tube is specific to the tube. You can use it as a checker marker or a coat button, but it does not serve these purposes well and it obviously finds its *specific* use in recapping the tube.

The front door of your house is much less specific. It is a windy, winter day and you leave the front door open; the newspaper blows off the hall stand; the temperature falls in the living room; the furnace goes on; the gas blows out on the kitchen range; two dogs wander in and sit on the davenport; the child strays into the street and is run over!

Now, you close the door. The house warms up; the furnace goes off; you can relight the gas stove and dispose of the dogs, but the marks they have left on the sofa and the dead child in the street are irreversible pieces of damage that cannot be corrected. You could ask a professor of logic for a sequence that would connect properly such diverse objects as newspapers, gas stoves, sofas, dogs and dead children, and he would be hard put to it to answer; yet, opening and closing a door has connected them all, all too logically. The front door is an important *non-specific conditioning* agent of your house. Vitamin E is the most important *non-specific conditioning* agent of the body. One may reflect in passing that in this fact lies the whole medical difficulty of linking clinical symptoms to vitamin E deficiency, or, rather, imbalance.

Now, let us introduce the "screw driver" hypothesis. You acquired the screw driver for putting in screws. Having it in the house, you have used it to jimmy up windows, open cans, dig weeds, and, in fact do anything that a screw driver will conveniently do. Any tool will be put to any use to which it is available, and, as a broad generalization, we may confidently state that any chemical tool will be put to any physiological use that the organism finds convenient. Is vitamin E a constituent of an enzyme? If it can be put to that use, we shall find it in that situation, with-

out prejudice to its other activities as specific or non-specific anti-oxidation, or as a building brick for sterol or hormone manufacture, or as a drug promoting endocrine activity!

In compiling a roster of things to look for, chemically, physiologically, and clinically, with vitamin E, we must remember that there are at least four tocopherols, their quinones, hydroquinones, and semiquinones, and it is a good bet that each one plays a part, large or small, in the total economy of the body.

THE ROLE OF FLAVONOIDS AND RELATED SUBSTANCES IN BIOLOGICAL OXIDATIONS¹

WILLIAM G. CLARK and T. A. GEISSMAN

*The Scripps Metabolic Clinic and the Department of Chemistry
University of California at Los Angeles*

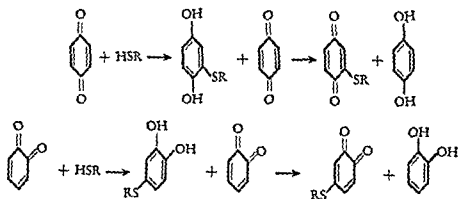
SINCE MATTILL (1) reemphasized the importance of the antioxidant properties of polyphenols containing *ortho* and *para* hydroxyl groups, many investigators have shown related action by such compounds.

The mechanism of phenolic antioxidant action depends upon the nature of the oxidation process and its catalysts, and therefore, may be of several different types. The most common type which has been studied is the simple hydrogen donor type in a homogeneous system, in which the antioxidant is preferentially oxidized in relation to the oxidant. The second most commonly studied type has been concerned largely with the postulated ability of these compounds to act as fat antioxidants by interference with the kinetics of the decomposition of hydroperoxides into free radicals which initiate chain oxidative processes. This also has been thought of as a preferential oxidation of the antioxidant alone; by oxidation in combination with a catalyst, or by removal of a catalyst such as cupric ion, which promotes the formation of reactive semiquinones.

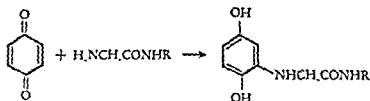
In the living organism, the *o*- and *p*-dihydroxybenzenes may act as "antioxidants" by reaction with oxidative enzyme systems. Quastel (2), Hellerman and Perkins (3), Potter and DuBois (4) and Bartlett (5) have shown that *ortho*- and *para*-quinoid compounds, including certain flavonoids (5), may inhibit such enzyme systems by reaction with essential thiol groups, by a 1,4 addition of protein-thiol to quinone, rather than by a direct oxidation. Snell and Weissberger (6), Kuhn and Bein-

¹ Grants from the U. S. Public Health Service, and The California Fruit Experiment Station, Los Angeles

art (7), and Fieser and Turner (8), have studied the combination of sulfhydryl compounds with quinones, and have shown that the 1,4 positions of the α,β -unsaturated ketonic grouping of quinones form addition compounds with the thiol.

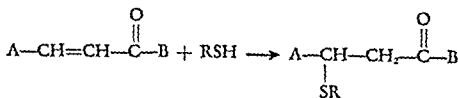


Other protein reactions also can occur with the quinones, as emphasized by Hoffman-Ostenhof (9,10), who points out that ever since Emil Fischer's work on the action of glycine ethyl ester with quinones, it has been known that amino groups of amino acid residues of proteins can react with quinones in much the same way as with thiol groups, thus:

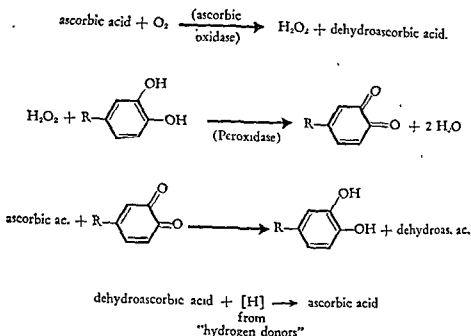


and that it is upon this principle that the quinone leather dye industry was based.

Some of the polyhydroxybenzene compounds including the flavonoids can react with thiol groups not through a quinoid mechanism but by virtue of the presence of an α,β -unsaturated ketonic grouping, as discussed by Posner (11,12) in 1902 and by Geiger (13,14), Cavallito, *et al.* (15,16) and others, in relation to biological systems. Like the linkage of RSH with the quinones, this occurs by a similar linkage of thiol to the ketone.



In addition to acting as biological antioxidants, some of the *ortho*- and *para*-polyhydroxybenzenes can act as *prooxidants*, as illustrated by the work of Huszak (17), who formulated the action of such substances as hydrogen transporters or coenzymes in the ascorbic oxidase-peroxidase-hydrogen peroxide system as follows



Bunatyan (18) also has observed acceleration of the oxidation of ascorbic acid by polyphenols in the presence of iron. Adrenalin was one such phenol.

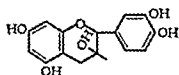
Lavollay and Neumann (19) described a similar acceleration by flavonoids of the oxidation of adrenalin by the system adrenalin-peroxidase-hydrogen peroxide. King had pointed out in 1939 (20) that this property is shared by many quinones, quinonimides, indophenols and other quinoid structures. It is interesting to note that Huszak found that the *o*-dihydroxyflavonoids were better hydrogen transporters than pyrogallol, hydroquinone and catechol.

Bacq (21,22) first demonstrated in 1935 that *ortho*- and *para*-polyhydroxybenzenes may act as biological antioxidants in the intact animal. He found that hydroxyhydroquinone, hydroquinone, catechol and pyrogallol potentiate and prolong the responses *in vivo* to adrenalin and sympathetic nerve stimulation. Resorcinol, phloroglucinol and similar *meta*-hydroxylated compounds were

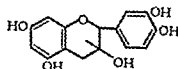
inactive. Bacq presented pharmacological evidence that the mechanism of this action is either by sensitizing receptors to adrenalin and "sympathin", or by inhibition of the enzymatic destruction of adrenalin at these tissue receptors; and that no effect was exerted on the level or rate of disappearance of adrenalin in the circulating blood itself.

Clark and Raventos (23) confirmed these observations in the case of pyrogallol, and both Bacq and these authors suggested that amine oxidase inhibition by the antioxidants explained the results. Richter (24), however, suggested that their results might be explained by substrate competition of the hydroxybenzene antioxidants with adrenalin for "sulfosynthase", which conjugates adrenalin as an ethereal sulfate.²

Parrot and Lavollay and their coworkers (26-31), following up these studies, proposed that the flavonoids may act as "vitamin P" in their anti-capillary fragility and anti-hemorrhagic effects, by similarly potentiating the action of adrenalin and sympathin in the body. They believe that of the flavonoids studied, *d*-epicatechin, a flavane, is most active. The catechins and epicatechins are illustrated below, and occur as both the *d* and *l* forms.



d- or *l*-catechin



d- or *l*-epicatechin

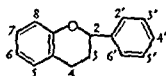
The adrenalin potentiation theory of Lavollay and Parrot has been supported by the observation of Fuhrman and Crismon (32) that the capillary bed vasoconstrictor threshold to adrenalin is lowered by intravenously administered rutin.

At this point it might be well to illustrate, by way of review, some of the structural relations of the flavonoid pigments (Fig. 1).

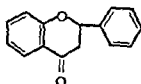
Since one of the most conspicuous pharmacological effects of the flavonoids is the potentiation of the depressor response of mammalian smooth muscle to sympathomimetic amines, Clark and Geissman (33,34,35) made a systematic study of the relation of structure to activity in this respect.

The test method has been described in detail elsewhere (35). It

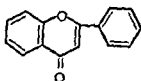
²Dodgson, Garton and Williams (25) recently have shown that adrenalin may be inactivated by conjugation as a glucuronide rather than as a sulfate.



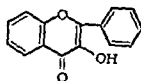
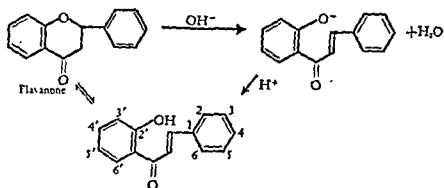
I. Flavane



II. Flavanone



III. Flavone

IV. Flavanol
(3-hydroxyflavone)

V. Chalcone

FIGURE 1

involved determining the prolongation of the relaxation of isolated rabbit intestinal (ileum) segments by adrenalin in the absence and presence of various compounds, using rutin as a reference standard and expressing the potentiation of the adrenalin response in terms of this standard ("rutin units"). Traces of cupric ion in the saline solution (Tyrode) were found to eliminate a certain lack of reproducibility of the results, presumably because of the presence of small but inconstant amounts of copper in the water, in the salts used for the saline mixture and in the substances tested. In the absence of these traces of cupric ion, there was a lack of reproducibility from test to test, and moreover, some of the more active

compounds prolonged the adrenalin response so much in the absence of added cupric ion that it became impossible to compare their activities.

The following compounds exceeded the activity of rutin four times or more on a mol equivalent basis: gossypetin, 3,3',4'-trihydroxyflavone, quercetin sulfonic acid, 8-hydroxyquinoline, quercetin, glutathione and 2',3,4-trihydroxychalcone.

The following had an activity of more than one but less than four times as great: sodium diethyldithiocarbamate, 3',4'-dihydroxyflavone, cysteine, gossypin, gossypitrin, butein, quercetin, esculetin, pyrogallol, and 3,4-dihydroxychalcone.

The following had the same activity as rutin: 2,3-dithiopropanol ("BAL"), cyanin-chloride, rutin (standard unit), leptosin and nordihydroguaiaretic acid ("NDGA").

The following had about half the activity of rutin: 3',4'-dihydroxyflavanone, *d*-catechin, *l*-epicatechin, epimerized *d*-catechin, 3-hydroxy-3',4'-dimethoxyflavone, chlorogenic acid, 2',3',4',3,4-pentahydroxychalcone, 5-hydroxyflavone, ascorbic acid and xanthorhamnetin.

Of negligible activity were: eriodictyol, 4,4'-dihydroxychalcone, disalicylalethylenediamine, 3,5,7,3',4'-pentahydroxyflavanone, hesperetin, 7,8,3',4'-tetrahydroxyflavanone, dihydroesculetin, phloracetin, disalicyl-*o*-phenylenediamine, esculin, rutin acid phthalate, rutin acid succinate, 3,4'-dihydroxy-4-methoxychalcone-4'-glucoside, sodium- α -tocopherol phosphate, 2'-hydroxychalcone, 2',4',6',3,4-pentamethoxychalcone, hesperidin, hesperidin acid phthalate, hesperidin acid succinate, "methylated hesperidin chalcone", "acetylated hesperidin chalcone", "hesperidin-3'-ethylcarboxylate", naringin, *o*-hydroxyacetophenone, 4-methoxy-3,2',4',6'-tetrahydroxychalcone-4'-glucoside, 4'-aminochalcone glucoside, 3,4'-dihydroxy-4-methoxychalcone-4'phosphate (disodium salt), butrin and inositol.

"Citrin", prepared according to Szent-Györgyi and modifications thereof, could not be assayed on a mol equivalent basis because of its unknown structure, but assuming a 70 per cent purity and that it may be eriodictyol rhamnoside (36), it has an activity of 0.2 that of rutin.

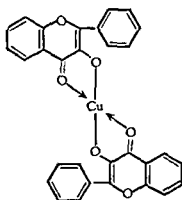
Pomiferin and gossypol acetate were too insoluble. Catechol, hydroquinone and resorcinol accelerated the destruction of adrenalin.

A spectrophotometric method was developed for studying the copper-catalyzed autoxidation of adrenalin to adrenochrome *in vitro*, but the results did not parallel the bioassay results; hence

manometric studies of the oxidation *in vitro* were not studied. These results are reported elsewhere (35).

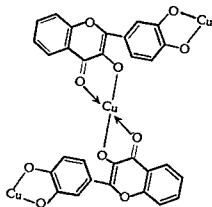
The most active substance was gossypetin, a flavone with an activity of 16.0 times that of rutin, on a weight basis. This was followed closely by 3,3',4'-trihydroxyflavone (15.5) and quercetin (10.0).

An examination of the series above shows that the most common structural features of the active substances is a grouping capable of forming complexes with heavy metals, and for those pairs of substances with closely similar structures, the more active forms a more stable complex. That chelation with metals occurs can be shown qualitatively by the formation of colored solutions or precipitates with metal ions; and by polarographic studies, which show disappearance or diminution of the normal copper wave and appearance of a new wave (the chelate) at a more negative half-wave potential in mixtures containing cupric ion and a number of the active compounds. Recently (37) the copper complex with flavonol (3-hydroxyflavone) has been isolated and purified, and its elementary analysis found to be in accord with the following structure.



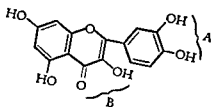
VI

Presumably 5-hydroxyflavone would act similarly, since it shows similar behavior in polarographic studies. Where the 3',4'-dihydroxy groupings exist, as they do in the highly active 3,3',4'-trihydroxyflavone, evidence obtained so far (37) indicates the complex,



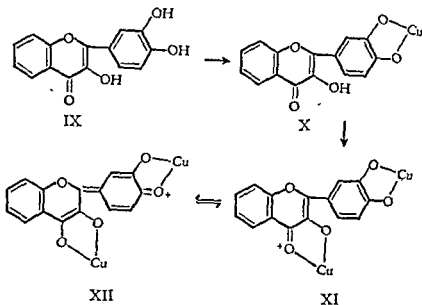
VII

The greater activity of quercetin compared with rutin, together with other considerations of the activity-structure relationships, led to the conclusion that in the flavonoid compounds, the important complex-forming elements were the 3',4'-dihydroxy groupings, and the 3-hydroxy, 4-keto groupings, shown as A and B in the structure for quercetin.



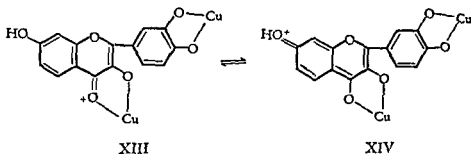
VIII

The importance of the 3-hydroxy grouping is illustrated below, in which the contribution of the second chelatogenic grouping to the resonant hybrid forms is shown.



The presence of the 2,3 double bond in the *flavones*, as contrasted with the *flavanone* structure, explains the higher activity of the former, since this double bond stabilizes the metal complex by contributing to the resonant hybrid forms shown in XI and XII above.

The presence of other, non-chelatogenic hydroxy groupings interferes with the resonating structures because of cross conjugation, and this decreases the stability of the complex. The interference by cross conjugation is shown below.



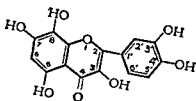
it being considered that the stabilization of the complex represented by the resonance hybrid forms XI and XII would be reduced by contributions from forms such as XIII and XIV.

From such considerations it was predicted early in the work that 3,3',4'-trihydroxyflavone would form a more stable complex than quercetin, with its interfering 5,7-hydroxyl groupings. Accordingly this compound was synthesized, tested, and the results found to confirm the prediction.

Similar arguments led to the study of 2',3,4-trihydroxychalcone, which was predicted to be more active than butein (2',4',3,4-tetrahydroxychalcone), a naturally-occurring substance. Butein contains, in addition to the chelatogenic 2'-hydroxyl group, a non-chelatogenic 4'-hydroxyl group, which introduces cross-conjugation and thus decreases the stability of the complex. Accordingly, 2',3,4-trihydroxychalcone was synthesized, tested, and the results found to fit the prediction.

These interpretations remain to be substantiated by further experimental evidence of other kinds.

The results, along with the occurrence of high activity of 8-hydroxyquinoline and sodium diethyldithiocarbamate, support the view that the active flavonoids potentiate the adrenalin response by protecting it from copper-catalyzed autoxidation. This concept is not entirely novel, since Lavollay's early studies (26) led him to a similar view which, however, differed in interpretation from that which we are inclined to adopt. Lavollay suggested that compounds containing the catechol nucleus (*o*-dihydroxybenzene) acted as adrenalin antioxidants *in vitro* by entering into a complex composed of adrenalin + copper + inhibitor, within which the protective substances were preferentially oxidized. Gossypetin (XV) was the most active compound examined in the present series.



XV

In this structure the 5,7,8-trihydroxy grouping represents an extremely readily autoxidizable hydroquinone-like structure, and may exert a direct antioxidant effect in protecting adrenalin, in addition to its metal chelatogenic properties due to the presence of the 3,3',4'-trihydroxyflavone structure. On the other hand, in confirmation of Lavollay, catechol, hydroquinone and resorcinol were adrenalin *prooxidants* rather than antioxidants. It is possible that catechol and hydroquinone catalyze the autoxidation of adrenalin through the mechanism proposed by LuValle and Weissberger (38). On the other hand, pyrogallol was quite active

as an antioxidant, which is in accord with the results which Bacq (21,22) and Clark and Raventos (23) obtained *in vivo*. But where hydroquinone and catechol were adrenalin "prooxidants" in the isolated smooth muscle test, Bacq found them adrenalin potentiators *in vivo*. These discrepancies are further complicated by the fact that simple preferential oxidation cannot play a leading role, since the E_0 values are: adrenalin > catechol > pyrogallol > hydroquinone (39). As mentioned before, it is possible that the effects *in vivo* can be explained in part by substrate competition of these phenolic substances with adrenalin for an enzyme which normally inactivates adrenalin by conjugation as a glucuronide or ethereal sulfate, as suggested by Richter (24). Considerable more work remains to be done to explain the discrepancies mentioned above.

In the cases of most of the other flavanoids tested, however, the protective action probably results simply from immobilization of the cupric ion. This conclusion is supported by the wide range of activity of flavones all of which contain the catechol nucleus but which differ in other details; and by the protective action of such substances as 8-hydroxyquinoline and 5-hydroxyflavone, which would not be expected to act by the mechanism of Lavollay. Lavollay assumed preferential oxidation of protector within a complex and although it is possible that a complex could form of adrenalin + Cu + 8-hydroxyquinoline or 5-hydroxyflavone, neither of the latter have groupings readily susceptible of oxidation.

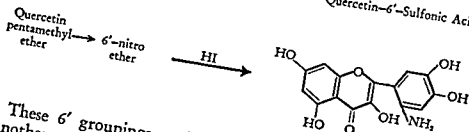
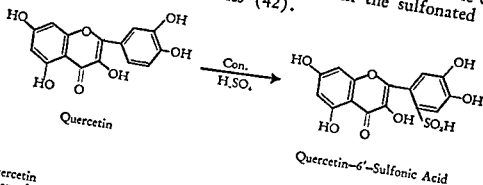
The wide differences in activity shown by substances having identical metal chelatogenic groupings, but differing in solubility characteristics, for example, such pairs as gossypin (soluble) and quercetin (insoluble); xanthorhamnetin (soluble) and rutin (less soluble)—supports this view, since in such pairs, the compound which is the more soluble exerts the lesser protective effect on adrenalin. This is in accord with what would be expected, a less soluble complex being more effective in immobilizing copper.

The results of Bacq, of Clark and Raventos and of Lavollay and Parrot, who found enhancement *in vivo* of adrenalin responses by such substances as catechol, pyrogallol and epicatechin, suggest that certain compounds of the kind used in this study might be capable of enhancing adrenalin responses in the intact animal by action at tissue sites. It is not possible, on the basis of our results to date, to regard the effects *in vivo* observed by these workers, and the gut segment responses reported in the present study, as being manifestations of a common type of action. Elsewhere (35), we have reported a lack of enhancement of a few

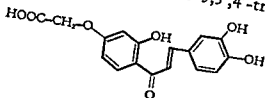
adrenalin effects *in vivo* by various flavonoids, but the methods used may be subject to criticism.

The fat antioxidant properties of flavonoid pigments have been described by several workers in the past, most recently by Richardson *et al.* (40) in their studies of milk, butter fat and lard. (They also review the literature on the subject). Their studies show a strong inhibition of the autoxidation and metal-catalyzed oxidation of these fats by flavonoids, especially quercetin.

It is possible that fat-soluble derivatives of the more active substances elucidated above will be found to have potent fat antioxidant properties, possibly surpassing such preparations as the esters of gallic acid, nordihydroguaiaretic acid (NDGA) and lauryl dithiopropanate. Quercitrin itself is not as effective as NDGA as a fat antioxidant used as a preservative of walnuts (41), but like its aglucone quercetin, this glycoside is only very slightly soluble in water, and hardly at all in fat. Fat soluble derivatives could be made, for example, from the sulfonated or nitrated derivatives of flavones (42).

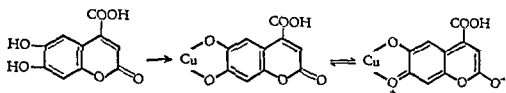


These 6' groupings could be substituted by lipophilic chains. Another possibility is to prepare 2',3,4-trihydroxy, 4'-carboxy-chalcone, or the related derivative of 3,3',4'-trihydroxyflavone,



the acid groups of which could be esterified with long chain fatty alcohols, or converted into long chain fatty amides.

Another possibility lies with esculetin-4-carboxylic acid, which has shown to have high activity and good water solubility, and which is capable of forming a stable metal complex.



The acid group here too could be esterified. In none of these examples would the lipophilic constituent interfere with the chelogenic groupings present.

An interesting "antioxidant" effect of the flavonoids in the intact animal has been described by the Chilean workers, Cruz-Coke and Reyes (43,44) who claimed a marked thiouracil-like, anti-thyroid effect of quercetin administered to rats. It was proposed that the mechanism of the action is by an "antioxidant" inhibition of iodide ion to iodine and its subsequent incorporation into diiodotyrosine. In a few preliminary experiments we have been unable to confirm these observations in rats, with quercetin, quercetin sodium sulfonate or rutin.

Another biological "antioxidant" action of the polyhydroxybenzene flavonoid-like compounds concerns that of the "sparing" of ascorbic acid in the animal body. Cotereau, Gabe and Parrot, (45,46,47), have shown that the thyroid activation in guinea pigs on a flavonoid-deficient, scorbutogenic diet, is prevented by ascorbic acid only if supplemented with the flavane, epicatechin. Large doses of epicatechin perform the same function in normal animals (indirect confirmation of Cruz-Coke and Reyes), and decrease the response of the thyroid to thyrotropic hormone.

The same authors (48,49) demonstrated that the adrenal cortex hypertrophy of animals on the flavonoid-deficient, scorbutogenic diet, is inhibited by small amounts of ascorbic acid only in the presence of epicatechin.

On a flavonoid-deficient diet supplemented with ascorbic acid, the ascorbic acid stores of liver and kidney were claimed by Cotereau *et al.* (50), to be markedly increased by the administration of epicatechin. Three different methods of ascorbic acid analysis were used. The following data is taken from their results, the figures representing mgm. per cent ascorbic acid

	Controls	Epicat. 1 mg./day	Ascorbic 10 mg./day	Both
Liver	1.9	2.9	3.4	20.0
Spleen	3.4	3.8	6.2	18.2
Kidney	3.8	1.4	2.0	20.3
Adrenals	6.7	10.2	5.0	33.5

Diet: Oats and bran 1:1; vit. B₁, B₂, niacin and cod liver oil for 23-26 days. Tillman's method. This work has not been supported by investigations conducted by Randall (51).

Most workers, including Parrot *et al.* themselves (52), have been unable to demonstrate any effect of flavonoids on survival or scorbutic symptoms of guinea pigs on acute or chronic scorbutogenic diets, although Ambrose (53) has claimed some ascorbic acid sparing action by massive oral doses of rutin and quercetin. Smaller doses, more comparable with the expected ingestion from a nutritional standpoint, have no such effect. This is illustrated by some of our own studies, in which a diet low in flavonoids (54) was fed for a month along with adequate ascorbic acid, followed by subminimal ascorbic acid (0.25 mgm. per animal per day) with and without 10 mgm. per day oral administration of 10 different representative flavonoids. Figure 2 shows the average body weight changes of the ten groups. Scorbutic symptoms paralleled the weight changes.

It was suggestive from these results that "Citrin" and esculetin might have had a slightly beneficial effect. Accordingly, another experiment was conducted, using these compounds, with essentially negative results.

Thus in the "normal" animal, it is improbable that the flavonoids function as essential nutrilites. Several investigators feel, however, that it is possible that a dietary essential may play a rôle only under conditions of stress, particularly in certain infections, sensitization or inflammatory conditions.

Other situations in which the flavonoids might play a nutritional rôle are those in which there is an increased demand for ascorbic acid, such as in trauma and wound healing (55,56,57); in the detoxication of certain drugs (58-62); and in high protein diets (63-66).

Regardless of the equivocal evidence that flavonoids may normally be essential nutrilites, Parrot still proposes to call *d*-epicatechin "vitamin C₂"; which is reminiscent of the first studies of the antiscorbutic vitamin, especially by Radoin and Lecoq (67), Bezssonoff (68), and Scotti-Foglieni (69), as early as 1926, when

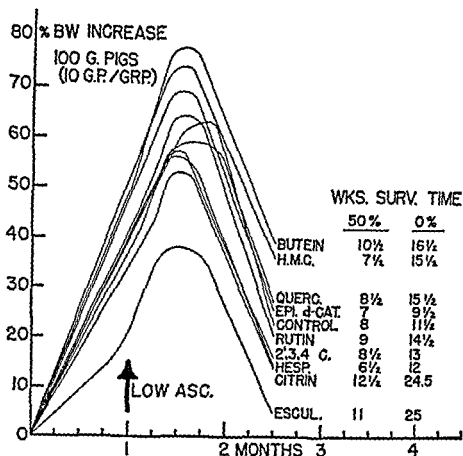


FIGURE 2. Effect of 10 mg. daily oral doses of 9 different flavonoids on survival and body weight of guinea pigs on a flavonoid-deficient diet and supplemented with daily subminimal amounts of ascorbic acid.

Key	
H.M.C.	"Methylated hesperidin chalcone"
Querc.	Quercetin
Epi. d-Cat.	Epimerized d-catechin
2',3,4 C	2',3,4-trihydroxychalcone
Hesp.	Hesperidin
Escul.	Esculetin

it was proposed that there existed heat stable and heat labile anti-scorbutic factors.

That the flavonoid type polyhydroxybenzenes are excellent ascorbic acid antioxidants *in vitro* has been observed by many workers, as well as the converse, that is, that these polyphenols are protected from autoxidation by ascorbic acid. Parrot and Cotureau (70) suggested that a sparing or antioxidant action occurs in the alkaline medium of the intestine. The antioxidant

action *in vitro* may be due to immobilization of minute traces of cupric ion. In this connection it is interesting to note that of the many antioxidants described for ascorbic acid in the literature, only the metal chelatogenic compounds such as 8-hydroxyquinoline which form highly stable metal chelates, inhibit the oxidation of ascorbic acid by both ascorbic acid oxidase and by copper-catalyzed autoxidation; while other retarders such as purines, amino acids, pyrimidines, thiols and disulfides, inhibit only the cupric-ion-catalyzed autoxidation (71). This probably is because the copper of the metalloprotein ascorbic oxidase is bound more tightly than in complexes with the ineffective retarders, but not as tightly as with 8-hydroxyquinoline, diethyldithiocarbamate, and the like.

Such a concept fits the recent provocative discussions of Albert *et al.* (72,73,74) who have related the antibacterial activity of chelatogenic oxines to the stability of their metal complexes, and who have suggested that the mechanism is by combination and hence inactivation of an ionized heavy metal forming part of metalloprotein enzyme molecules.

Chelatogenic compounds may have very interesting theoretical and practical applications in the study of the metabolism of trace metals. For example, hydroxyacridines, aminohydroxyacridines, oxines, flavonoids and other chelators may exert their known antibacterial, fungicidal and pesticidal activity by immobilization of essential heavy metals. Recently McCance and Widdowson (75), McDonald (76) and Trunnell *et al.* (77), have shown that intravenously administered BAL-glucoside in man and sheep, increases the urinary excretion of endogenous copper up to twenty fold, and that of zinc five fold. It is interesting to consider the possibility that the metal chelating compounds may be able to perform similar functions. The observations that flavonoids exert a protective action against arsenic poisoning (61,62) is suggestive of such possibilities.

Michaelis earlier in this conference already has discussed the interesting properties of the oxygen-carrying chelates in relation to the action of respiratory pigments.

An antibacterial action *in vivo* was described recently by Goth and Robinson (78), which has been ascribed to chelation with metals essential for the invading microorganisms. These authors found that certain dithiocarbamates protected mice against otherwise fatal injections by pneumococci, although the doses required were high. Bismuth synergized the effect. It is possible that important applications may be made of some

of the concepts discussed above, to studies *in vitro* and in the intact organism, of the biological rôle of essential metals such as cobalt, zinc, copper, and iron. It is also provocative to consider the potentialities of such substances in controlling the therapeutic uses of arsenic, gold, tellurium, antimony, bismuth, etc.; and in the control of radiation therapy from more or less organ specific isotopes such as zinc, gold, chromium, arsenic and cobalt.

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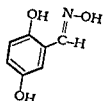
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DISCUSSION

Weissberger: Dr. Clark, your mentioning that gossypetin might be the most active compound because it is both chelatogenic and quinoid in nature, suggests to me that gentisaldehyde,



also might have these two properties within the same molecule. It is a very powerful precipitant of copper.

W. G. Clark: We would like to examine it.

Michaelis: Have you compared the hydroquinone dyes with your series?

W. G. Clark: A few were tested, but not many. No quantitative comparisons were obtained.

György: What is the toxicity of some of the substances you tested?

W. G. Clark: The toxicity of parenterally administered rutin is very low. Quercetin sodium sulfonate, one of the most active in the series, has a toxicity of 150 mg./Kg., intraperitoneally in rats.

Seifter: Adrenalin-potential is a very non-specific phenomenon, shared by such diversified drugs as cocaine, ephedrine, yohimbine, antihistamines, etc.

W. G. Clark: I realize this; we have restricted our attention to a particular molecular species, and it would be interesting to see if the *in vitro* results we have obtained would show the same trend *in vivo*, in regard to adrenalin potentiation.

György: What amounts of copper are inactivated by the flavonoids examined?

W. G. Clark: According to the copper complexes which Geissman has isolated and studied, it can be predicted exactly how much copper will be removed by certain flavonoids, depending on the structure of the particular flavonoid used. As shown in our paper above, 3-hydroxyflavone combines with copper in the ratio of two molecules per atom of copper; and 3,3',4'-trihydroxyflavone in the ratio of 2 moles per 3 atoms of copper. Chelatogenic compounds have indeed been used to remove trace

heavy metals from bacteriological media, for example, Waring and Werkman (*Arch. Biochem.*, 1, 303, 1942), have used 8-hydroxyquinoline to remove iron from bacterial media in order to study the necessity of iron for bacterial growth. The excess quinoline is removed by extraction with chloroform. Adrian Albert, *et al.* have confirmed this (*Brit. J. Path.*, 28, 69, 1947).

The whole point of Albert's papers was to stimulate the search for greater metal specificity in chelatogenic compounds, so that they would be more useful to biologists. For example, if we had a substance which was specific for cobalt, it might be very interesting as a tool in studying vitamin B₁₂, which is a cobalt complex itself.

REPORT ON SYMPOSIUM DES LIPIDES¹

RICHARD H. BARNES
Sharp and Dohme, Inc.

DURING January of 1948, a symposium on lipids was held at the Sorbonne in Paris under the sponsorship of the Centre National De La Recherche Scientifique. Twenty-four members representing France, Switzerland, Holland, England and the United States participated. Broad fields of fat metabolism including digestion and absorption, deposition and mobilization, *in vivo* and *in vitro* oxidation, lipoproteins, and antioxidants were discussed. Outstanding among the contributions were studies from the Laboratory of Mademoiselle Le Breton of the Sorbonne on the role of cholesteroesterase and lecithinase in fat metabolism, studies on tri-glyceride absorption from the intestines from the Birmingham Laboratories of Professor Frazer and the lipoprotein studies of Professor Chargaff of New York. Much of the work presented at this symposium was in the way of review.

The autoxidation of fatty acids and the role of certain antioxidants in this type of fat oxidation has been under investigation by several groups of workers in France. Professor Chevallier of the University of Strasbourg discussed his experiments on light catalyzed oxidation of fatty acids as measured by ultra-violet absorption spectra. Professor Paquot of Paris discussed the autoxidation of saturated fatty acids, as compared with unsaturated fatty acids.

Unfortunately, the French workers have not had access to a great deal of the scientific literature dealing with fatty acid oxidation and, in addition, they have not had access to pure fatty acid preparations. The results of their investigations were interesting, but many conclusions that were drawn were not valid in the light of present day knowledge.

One casual observation reported by professor Chevallier may

¹ Held in Paris, France, January 5-12, 1948.

have significance in our discussion of biological antioxidants. In his studies on light catalyzed fat oxidation, either guinea pig or rabbit adipose tissue fat has been used for the reason that fat from these species has a very short induction period. This suggests that natural antioxidants are not present in appreciable amounts in the adipose fat, which, of course, is contrary to observations that have been made in either species. This may have some bearing on the ease of development and type of reaction that is caused by vitamin E deficiency in the rabbit, as compared with the rat.

DISCUSSION

Lundberg: I should like to ask whether the French workers found that any peroxides were formed in the oxidation of the saturated fatty acids. It has been found by people in this country that esters of palmitic and stearic acids, when left in contact with oxygen for long periods of time, gradually develop peroxides which are apparently hydroperoxides.

Barnes: Well, I can't answer you very well. The only stress given to hydroperoxide formation was in the unsaturated fatty acid oxidations.

I don't know whether they found hydroperoxide formation in the saturated acids.

THE ANTIOXIDANT EFFECT OF ESTROGENS AND ANDROGENS¹

ROLAND K. MEYER and W. M. McSHAN

Department of Zoology, University of Wisconsin

THE DATA to be discussed in this paper are a part of those obtained from investigations which were designed to study the manner in which hormones produce their profound effects. The data to be presented are concerned principally with the *in vitro* effect of natural and synthetic estrogens and androgens on succinic dehydrogenase, malic dehydrogenase and cytochrome oxidase of rat tissues (1, 2, 3).

EXPERIMENTAL

The tissues were obtained from rats of the Sprague-Dawley strain. The animals were killed by cervical dislocation and the tissues prepared for study by the homogenization method of Potter and Elvehjem (4). The homogenates for the succinic dehydrogenase or cytochrome oxidase determinations were made in concentrations of 1% for heart and kidney, and 5% for all other tissues, including liver. Two and one-half per cent homogenates of liver were used in the malic dehydrogenase studies. The method used for determining the succinic dehydrogenase activity of the tissue was that described by Potter (5) and Potter and Schneider (6). Malic dehydrogenase was studied by the method developed by Potter (7).

In experiments designed to ascertain whether the compounds inhibited succinic or malic dehydrogenase directly, or through cytochrome oxidase, use was made of the findings of Weil-Malherbe (8), who demonstrated that the succinoxidase system would function without cytochrome *c* in the presence of brilliant cresyl

¹ Supported by grants from the Research Committee of the University of Wisconsin, from funds of the Committee on Research in the Division of Research Grants and Public Health Service, and from the National Cancer Institute, U. S. Health, U. S.

blue, although not to the same extent as with cytochrome *c*. Another method described by Schneider and Potter (9) was also used to test whether cytochrome oxidase was involved. This method provides for the use of ascorbic acid as a substrate for cytochrome oxidase.

Since estrogens, androgens and related compounds are so insoluble in water it was necessary to devise a way to render them soluble to the degree necessary for the investigations. Solutions of diethylstilbestrol, hexestrol, dienestrol, the benzenestrols, sodium 3,4-diphenylhexane-p-hydroxy-p'-oxyacetate (oxyacetate of hexestrol) and the compounds listed in Tables V and VII were made by adding 0.3 ml. of water to the calculated weight of each compound, followed by 0.03 ml. of 2 M sodium hydroxide. After the substances were in solution a part of the sodium hydroxide was neutralized with 0.01 ml. of 2 M HCl, and sufficient water added to make a volume of 10 ml., which gave a 0.001 M stock solution of each of the compounds. The requisite amount of each solution was added to the Warburg flasks at the time the tests were made. Solutions containing equivalent concentrations of sodium hydroxide and hydrochloric acid were prepared and used in the control flasks.

Estrone sulphate, androsterone sulphate and 3-4-diphenylhexane-p-p'-dioxyacetate (dioxyacetate of hexestrol) were sufficiently soluble in water so that stock solutions of 0.001 M and 0.002 M concentrations could be made directly.

In the experiments conducted to determine whether the estrogens were inactivated when they inhibited the enzyme systems, the contents of the Warburg flasks were assayed in adult castrated rats. A mixture of boiled tissue with fortified substrate plus the estrogen was used as a control.

To determine whether estrogens inhibit the succinoxidase system *in vivo*, 25 mg. of diethylstilbestrol in oil were injected into adult female rats daily for 10 days. Liver and uterine tissues were removed on the 11th day and the activity of the succinoxidase system compared with that of the same tissues of uninjected rats.

RESULTS AND DISCUSSION

The data recorded in Table I show that, *in vitro*, diethylstilbestrol in concentrations of 0.2 to 2.0×10^{-4} M inhibited the succinoxidase system in the liver of rats. The inhibition became greater with increasing concentrations of the estrogen. The high-

TABLE I

Inhibition of the Succinoxidase System of Rat Liver by Different Concentrations of Diethylstilbestrol

Experiment No.	Control	QO ₂				
		Final molarity of inhibitor (10 ⁻⁴)				
		0.2	0.25	0.5	1.0	2.0
1	88.0	71.3	—	40.6	20.8	0.0
2	80.4	72.9	63.3	52.0	28.3	0.0
3	77.1	—	—	38.4	23.8	2.2
Average	81.8	72.1	63.3	43.6	24.3	0.7

TABLE II

Inhibition of the Succinoxidase System of Rat Tissues by Diethylstilbestrol

Kind of rats	Tissue	Dry weight	Homog. used ml.	No. of expts.	Qo ₂		
					Control	Molarity (10 ⁻⁴)	
						1.0	2.0
Adult	Brain	Per cent 21.3	5%				
			0.2	3	40.5	11.8	
			0.3	5	39.6	27.0	9.1
Adult	Adrenal	30.0	0.2	4	29.7		
			0.3	4	30.0	18.2	
Young males	Adrenal	30.0	0.2	2	41.2	10.7	
			0.3	3	42.9	31.7	
	Pituitary	21.3	0.2	2	20.0		1.0
			0.3	2	20.0	2.8	
Adult-preg. 12 days	Corpora lutea	22.1	0.2	1	35.9	4.8	
	Ovarian residue	20.6	0.2	1	15.2	0.0	
Adult	Kidney	23.7	1%				
			0.2	2	170.7		
			0.3	5	166.5	15.2	
			0.4	1	185.4	65.9	
Adult	Heart	23.1	0.2	5	167.2	9.4	
			0.3	7	162.2	14.4	
			0.4	3	153.3	36.6	

est concentration used, 2×10^{-4} M, gave complete inhibition in two experiments and almost as much in a third.

The succinoxidase system in the other tissues of the rat was also inhibited by diethylstilbestrol as demonstrated by the data presented in Table II. A noteworthy result was the very effective inhibition of kidney and heart enzyme systems by 1×10^{-4} M concentration of estrogen. The succinoxidase system in brain and adrenal tissue did not seem to be as effectively inhibited by diethylstilbestrol as that in the other tissues studied.

It became of interest at this stage of the study to determine whether other synthetic estrogens, a natural estrogen, and androgens would act as effective inhibitors of the succinoxidase system of rat liver. The results of these experiments demonstrate that hexestrol and dienestrol are approximately as efficient as diethylstilbestrol (Table III). Estrone sulphate, however, was not of the same order of efficiency as were the three synthetic estrogens (Tables III and IV).

TABLE III

Effect of Estrogens on the Succinoxidase System of Liver Tissue
Obtained from Adult Rats

No. of runs	Q_{O_2}			
	No inhibitor	Final molarity of inhibitor (10^{-4})		
	Control	1.0	2.0	4.0
9	82.9(65.6-106.3)	18.7(10.4-28.4)	Diethylstilbestrol —	—
5	76.6(57.3-86.4)	18.0(7.0-29.2)	Hexestrol —	—
3	87.1(67.4-106.3)	19.8(10.5-30.7)	Dienestrol —	—
5	81.7(63.3-99.8)	76.8(54.9-88.8)	Sodium 3,4-diphenylhexane- <i>p</i> -hydroxy- <i>p'</i> -oxyacetate 68.5(52.7-92.7) 47.5(51.1-43.9)	
4	86.1(78.7-89.8)	87.6(86.0-88.8)	Disodium 3,4-diphenylhexane- <i>p,p'</i> -dioxyacetate 85.3(78.1-88.9) 84.0(76.4-92.0)	
2	78.9(78.9-79.0)	69.2(68.9-69.6)	Sodium estrone sulphate 64.8(60.9-66.9) 50.2(48.1-52.1)	

TABLE IV

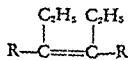
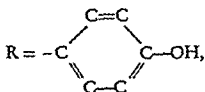
Effect of Sodium Sulphate Salts of Androgens and Other Compounds on the Succinoxidase System of Rat Liver

Compound	No. of expts.	QO ₂				
		Control	Final molarity of inhibitor (10 ⁻⁴)			
			0.5	1.0	2.0	3.0
Androsterone sulphate-sodium salt	3	71.2		70.4	71.6	71.8
Isodehydroandrosterone sulphate-sodium salt	3	71.2		70.0	65.0	73.6
Premarin*	4	67.3		60.6	55.3	
Sodium sulphate salt of estrone	4	74.1		60.2	54.2	

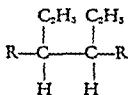
*An impure sample of sodium estrone sulfate supplied by Ayerst, McKenna and Harrison. The pure sample of sodium estrone sulphate was also supplied by the same company.

In contrast to the estrogens, the sodium salts of androsterone sulphate and isodehydroandrosterone sulphate did not inhibit the succinoxidase system of liver in a concentration of 3×10^{-4} M (Table IV).

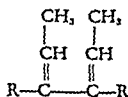
The fact that these synthetic estrogens have two phenolic groups, as shown by the following formulae, and estrone has one, suggests that the phenolic groups are the places in the molecule that are concerned with the antioxidant effect.



Diethylstilbestrol

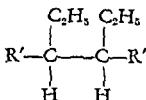
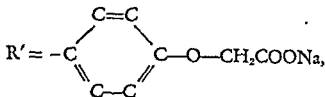


Hexestrol

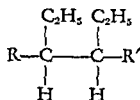


Dienestrol

This hypothesis is also supported by the data obtained with the sodium salts of the dioxyacetate and oxyacetate of hexestrol (Table III). The formulae of these substances are given below.



Disodium 3,4-diphenylhexane-*p,p'*-dioxyacetate



Sodium 3,4-diphenylhexane-*p*-hydroxy-*p'*-oxyacetate

The latter, which has one free phenolic group, is an effective inhibitor; whereas the former, which has both hydroxyl groups replaced by oxyacetate groups, did not inhibit. Estrone, with one phenolic group had approximately the same antioxidant effect as hexestrol oxyacetate.

The relationship of the phenolic groups to the inhibition of the succinoxidase system of liver was studied further by investigating a series of phenolic diesters of stilbene compounds (Table V). All of these compounds inhibited, but diethylstilbestrol distearate was not as effective as the others. Although the phenolic hydroxyl groups in these compounds were replaced by propionic and stearic acid ester groups the compounds did not lose their inhibitory power, which they did when the hydroxyl groups of hexestrol were replaced with oxyacetate groups. This difference might be explained by assuming, without direct evidence, that there was an esterase in the liver homogenate which caused the free estrogen to be liberated, which then acted on the succinoxidase system. The oxyacetates being more stable do not yield free hexestrol.

Thus far in our discussion we have been concerned with compounds which are among the most potent of the estrogens. The data from these compounds indicate that the estrogenic activity can be correlated with the antioxidant activity, and the latter effect, in turn, with the presence of free phenolic groups. When we extended our study to the six benzestrols listed in Table VI, we

TABLE V

The Effect of Phenolic Diesters of Stilbene Compounds on the Succinoxidase System

No. of compound	Name of compound	Q ₀₂		Inhib. %	Solubil. sty***	Approximate estrogenic activity
		Control (without compound)	Experimental (with compound)*			
107	Diethylstilbestrol dipropionate	82.7(7)**	21.1(8)**	74	S	gamma/r.u. 1.0
109	Diethylstilbestrol distearate	82.8(6)	60.3(6)	27	PS	<50.0
111	Hexestrol dipropionate	78.8(6)	32.2(6)	60	PS	1.6
115	Dienestrol dipropionate	80.8(6)	40.4(6)	50	S	2.6

* Concentration of compounds, 1×10^{-4} M

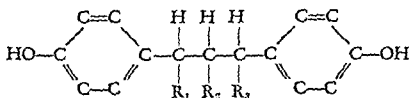
** Number of flasks used in making determinations.

***S, soluble; PS, partly soluble.

dipropionate 80.8(6) 40.4(6) 60 S 1.6

TABLE VI

Inhibition of the Succinoxidase System of Rat Liver by Benzeestrol Compounds



Formula of benzeestrol type compounds

Number Compound	R ₁	R ₂	R ₃	Estrog. activity mg./RU	No. of runs	Q ₀₂		
						Control (no inhib.)	Final molarity (10 ⁻⁴)	
							0.5	1.0
020	H	C ₂ H ₅	H	5.0	5	73.0	50.6	23.3
103	CH ₃	H	C ₃ H ₇	0.2	6	72.4	30.5	3.5
22B	C ₂ H ₅	C ₂ H ₅	H	0.04	4	73.2	42.4	5.5
32B	C ₃ H ₇	C ₂ H ₅	H	0.2	5	73.3	20.5	1.1
231B-2	C ₂ H ₅	C ₃ H ₇	CH ₃	0.005	4	79.4	30.9	2.0
221B-2	C ₂ H ₅	C ₂ H ₄	CH ₃	0.0008	6	76.0	23.0	1.3

found that although two free phenolic groups are present in all six compounds the antioxidant activity was not alike for all compounds. Thus, compound 020 was not nearly as effective as compound 221B-2, although both are diphenols. Furthermore, no very precise correlation exists between estrogenic activity and antioxidant effect, although the weakest estrogen (020) was also the weakest inhibitor of the succinoxidase system and the strongest estrogen (221B-2) was one of the strongest inhibitors. Recently Case and Dickens (10) have reported that some non-estrogenic substances strongly inhibit the succinoxidase system, while other highly potent estrogens are almost completely devoid of inhibitory effects.

The study was extended to the investigation of a number of non-phenolic compounds which were available (Table VII). As a group these compounds had little, if any, estrogenic activity

TABLE VII

The Effect of Nonphenolic Compounds on the Succinoxidase System

No. of compound	Name of compound	Q ₀₂		Inhib %	Solubility***	Approximate estrogenic activity
		Control (without compound)	Experimental (with compound)*			
69	1-Keto-1,2,3,4-tetrahydrophenanthrene	84.1(6)**	60.6(6)**	28	PS	gamma/r.u. >5000
78	1,2-Bis-(p-methoxyphenyl)-cyclohexene	79.8(8)	52.5(8)	34	PS	>1000
80	9-(α -Chloroanisal)-xanthene	75.7(6)	49.5(6)	35	PS	>1000
81	p,p'-Dimethoxy- α,α' -diacetoxystilbene	79.8(6)	73.8(6)	8	I	>3000
83	4,4'-Dimethoxybenzal-aniline	71.2(6)	66.3(6)	7	PS	>1000
91	Tri-(p-methoxyphenyl)-ethylene	84.6(6)	70.2(6)	15	PS	12000
95	Dianisalacetone	76.8(6)	69.6(6)	10	PS	1000
119	Triphenylethylene	82.0(6)	27.1(6)	67	S	>500

*Concentration of compounds, $1 \times 10^{-4} M$

**Number of flasks used in making determinations.

***S, soluble; PS, partly soluble; I, insoluble.

when measured by the vaginal smear method in the rat, and they were not completely soluble in the media used for determining the activity of the succinoxidase system. Unfortunately the degree to which a compound is soluble in an aqueous medium determines its effectiveness as an inhibitor. This should be kept in mind when considering the data in Table VII.

It will be seen that compounds 69, 78, 80 and 119 had appreciable antioxidant effects, particularly 119 (triphenylethylene). None of these compounds have phenolic groups and as a whole they could never be more than very weak estrogens. The effective antioxidant action of triphenylethylene is of interest since it is not a phenolic compound and has only weak estrogenic properties when administered subcutaneously. This inhibitor effect, however, becomes more understandable when one considers that triphenylethylene is a proestrogen (11), that its estrogenic activity is potentiated by passage through the liver (12), and that it is believed to be converted to a phenolic type of estrogen by the liver (12, 13). This latter possibility is strengthened by the isolation of phenolic estrogens from urine after the injection of phenyl proestrogens (14). On the basis of this evidence it does not seem unreasonable to suggest that triphenylethylene *in vitro* might become converted by liver homogenates to a phenolic substance which would inhibit the succinoxidase system.

Recently a method has been devised for determining the activity of malic dehydrogenase in animal tissues (11) and it became of interest to ascertain whether estrogens would inhibit this enzyme system. The data presented in Table VIII demonstrate that the synthetic estrogens (diethylstilbestrol, hexestrol, dienestrol) and compounds 103 and 221B2 of the benzestrol series are effective inhibitors of malic dehydrogenase in rat liver. Disodium 3,4-diphenylhexane-p-p'-dioxyacetate (dioxyacetate of hexestrol), benzestrol 020 and sodium androsterone sulphate have little or no antioxidant action on the malic dehydrogenase system. It is to be emphasized that the presence of two oxyacetate groups in hexestrol causes a marked decrease in the inhibitory action of this compound on the malic dehydrogenase system as was found to be the case for the succinoxidase system.

It was first assumed that the mechanism of the antioxidant effect of the potent synthetic estrogens was due to the inhibition of the succinic dehydrogenase of the succinoxidase system, i.e., similar to the inhibition of the enzyme by malonate. The fact, however, that the cytochrome *c* in the flasks with the diethylstilbestrol was observed in some cases to be in the reduced state immediately

TABLE VIII

Malic Dehydrogenase Activity of Liver with Estrogenic and Androgenic Inhibitors *in Vitro*

Q ₀₂		Inhibitor	Inhibition, %	Avg., %
Control	Final molarity 10 ⁻⁴ M			
		Diethylstilbestrol		
55.9	22.5		60	44
79.7	46.9		42	
82.6	57.3		30	
		Hexestrol		
71.8	21.3		70	75
75.3	26.6		81	
		Dienestrol		
90.7	60.3		34	36
90.8	34.2		63	
64.0	42.7		33	
65.8	38.4		32	
79.2	47.4		40	
80.6	74.8		7	
80.6	48.2		40	
		Disodium 3,4-diphenylhexane-p,p'-dioxyacetate		
64.0	61.4		4	6
65.8	59.7		9	
76.5	77.5		0	
85.4	76.2		11	
		No. 020		
84.2	65.2		23	22
78.3	62.6		20	
		No. 103		
84.2	4.8		94	93
78.3	6.1		92	
		No. 221B2		
90.7	10.4		89	90
90.8	8.0		91	
		Na Androsterone SO ₄		
79.2	75.0		6	2
80.6	80.9		0	
80.6	80.9		0	

after the tests were completed indicated that cytochrome *c* was not being oxidized. That suggested that the antioxidant effect might be mediated through cytochrome oxidase. This hypothesis would explain the inhibition of both the succinic and malic systems by estrogens since both of these dehydrogenases function through cytochrome oxidase.

The hypothesis just formulated was tested by the use of brilliant cresyl blue in place of cytochrome *c* in the media used for testing the activity of malic and succinic dehydrogenases. This dye is autoxidizable and permits the dehydrogenases to function in the absence of cytochrome *c*. Thus, if the inhibition by the compounds is effected by combination with cytochrome oxidase, the oxygen uptake should be approximately the same both in the presence and absence of inhibitor. The results for phenolic estrogens, which are given in Tables IX, X and XI, show that this was the case since, when the dye was used with or without estrogen, the

TABLE IX

Effect of Brilliant Cresyl Blue on the Inhibition of the Succinoxidase System of Liver by Diethylstilbestrol

Sod. succ. 0.5 M	Cytochrome c $3 \times 10^{-4} M$	Brilliant cresyl blue 0.5%	Diethylstil- bestrol $10^{-3} M^*$	I	Qo ₂ Experiments II	III
ml.	ml.	ml.	ml.			
0.3	0.2	—	—	77.5	53.7	68.0
0.3	0.2	—	0.3	5.9	12.7	27.3
0.3	—	0.5	—	37.0	32.5	42.1
0.3	—	0.5	0.3	31.3	30.2	38.8

*Final concentration was $10^{-4} M$ when 0.3 ml. was used.

TABLE X

Effect of Brilliant Cresyl Blue on the Inhibition of the Succinoxidase System of Liver by a Benzestrol Type Compound

Sod. succ. 0.5 M	Cytochrome c $3 \times 10^{-4} M^*$	Bril. C blue 0.5%	Benzest. No. 103 $1 \times 10^{-3} M$	Qo ₂ Average
ml.	ml.	ml.	ml.	
0.3	0.2	—	—	69.4
0.3	0.2	—	0.3	2.8
0.3	—	0.5	—	32.3
0.3	—	0.5	0.3	27.0

*Final concentration in flasks was $2 \times 10^{-5} M$.

TABLE XI

Inhibition by Diethylstilbestrol of Malic Dehydrogenase of Liver Using Cytochrome c and Brilliant Cresyl Blue

Q _{o2}					
Cytochrome c		Cresyl blue		Cytochrome c-Cresyl blue	
Control	Inhibitor*	Control	Inhibitor*	Control	Inhibitor*
84.3	34.4	82.2	62.9	—	—
62.9	3.5	69.0	61.6	—	—
70.0	13.0	73.9	71.0	77.5	71.3
75.3	27.2	78.7	75.6	71.3	68.9
71.2	5.9	67.9	59.8	64.0	61.9
71.2	7.1	69.2	58.3	64.0	60.4
74.8	11.8	75.1	63.0	70.1	67.8
Avg 72.8	14.7	73.7	64.6	69.4	66.1

*Diethylstilbestrol, final concentration 1×10^{-4} M.

oxygen uptake was approximately the same in both cases. Actually the oxygen uptake was slightly less in most cases when the estrogen was used in the system. The data indicate that the antioxidant effect of the phenolic compounds used in this study is exerted principally on cytochrome oxidase rather than on succinic and malic dehydrogenase.

Further support for this theory was obtained when ascorbic acid was used as a substrate for cytochrome oxidase, with and without diethylstilbestrol. The Q_{o2} for those flasks with ascorbic acid, cytochrome c and liver tissue was 317 as contrasted with a value of 153 for those flasks containing ascorbic acid, cytochrome c, tissue, and diethylstilbestrol. These results are explained on the basis that diethylstilbestrol inhibited the oxidation of ascorbic acid by cytochrome oxidase.

The results given in Table XII were obtained by the use of brilliant cresyl blue with the non-phenolic compounds, including esters. These data show that those non-phenolic compounds (the esters in Table V, and 69, 78, 80, and 119 in Table VII) which inhibited the succinoxidase system appreciably, do not have any antioxidant effect when used with brilliant cresyl blue. This is also true for the non-phenolic compounds which inhibited the succinoxidase system slightly (Table XII). These data show that the non-phenolic compounds also exert their antioxidant effect through cytochrome oxidase.

TABLE XII
The Action of Nonphenolic Compounds on Cytochrome Oxidase

No. of compound	Name of compound	O ₂	
		Brilliant cresyl blue	
		Control (without compound)	Experimental (with compound)*
69	1-Keto-1,2,3,4-tetrahydro-phenanthrene	42.5(2)**	49.6(2)**
78	1,2-Bis-(p-methoxyphenyl)-cyclohexene	39.1(2)	38.8(2)
80	9-(α -chloroanisyl)-xanthene	45.8(2)	45.0(2)
81	p,p'-Dimethoxy- α - α' -diacetoxystilbene	39.0(2)	41.2(2)
83	4,4'-Dimethoxybenzalaniline	40.8(2)	42.0(2)
91	Tri-(p-methoxyphenyl)-ethylene	40.0(2)	40.4(2)
95	Dianisalacetone	45.8(2)	54.0(2)
119	Triphenylethylene	38.8(2)	41.9(2)
107	Diethylstilbestrol dipropionate	39.1(2)	41.1(2)
109	Diethylstilbestrol distearate	39.1(2)	40.4(2)
111	Hexestrol dipropionate	46.7(2)	46.8(2)
115	Dienestrol dipropionate	42.5(2)	41.6(2)

*Concentration of compounds, 1×10^{-4} M.

**Number of flasks used in making determinations.

In summarizing the information which we have presented we conclude that: 1) some phenolic synthetic estrogens exert a powerful antioxidant effect on the succinoxidase and malic dehydrogenase systems of rat liver, but the antioxidant effect is not directly correlated with estrogenic activity; 2) non-phenolic compounds as well as phenolic ones have this property, but not all of them to the same degree; 3) the antioxidant action exerted by the compounds studied is mediated mainly by the inhibition of cytochrome oxidase.

The results of Case and Dickens (10), which were published recently in abstract form, should be discussed in relation to conclusion 3. They report that some compounds which they tested on the succinoxidase system inhibited succinic dehydrogenase; other compounds acted on cytochrome oxidase, and still others on both the dehydrogenase and oxidase. They also found one compound, p'-p'-dihydroxystilbene, which inhibited the overall oxidation of succinate, but was without effect on either cytochrome oxidase or succinic dehydrogenase. Thus in the light of this report

it can not be stated categorically that all estrogens or compounds chemically related to estrogens manifest their antioxidant effect on the succinoxidase and malic dehydrogenase systems by acting only on cytochrome oxidase.

Thus far the discussion has been concerned mainly with the *in vitro* antioxidant effect of estrogens and the mechanism of this effect. It became of interest, however, during the course of the investigation to determine whether an estrogen having a powerful inhibitory action on the oxidation of succinate by liver tissue *in vitro* would manifest this effect *in vivo*. For this purpose intact adult female rats were given 25 mgm. of diethylstilbestrol in oil per day for ten days. On the day after the last injection the rats were killed and the succinic dehydrogenase and cytochrome oxidase activities were determined in samples of their livers and uteri (Table XIII). Although diethylstilbestrol caused marked growth

TABLE XIII

Effect of Diethylstilbestrol on Succinic Dehydrogenase and Cytochrome Oxidase *in Vivo*

Exp. no.	Treatment	QO ₂ of tissue			
		Liver		Uterine	
		Succ. dehyd.	Cyto.oxid.	Succ. dehyd.	Cyto. oxid.
1	None (control)	94.5	426.8	4.2	78.4
2		72.9	326.8	5.0	76.9
		Av. 83.7	376.8	4.6	77.6
1	Diethylstilbestrol*	72.4	372.4	6.0	83.4
2		65.7	405.4	5.0	63.3
		74.8	468.6	3.0	71.7
3		Av. 71.0	415.5	4.8	72.4

*Rats were injected with 25 mg. in oil per day for 10 days

of the uteri *in vivo* the activity of succinic dehydrogenase and cytochrome oxidase of these uteri was not significantly different from the controls. No *in vivo* action of diethylstilbestrol on the enzymes of the liver was obtained, but it is probable that the liver was inactivating a considerable portion of the diethylstilbestrol administered (12, 15). We did not, however, demonstrate any *in vitro* inactivation of either estrone sulphate or diethylstilbestrol

TABLE XIV

Assay of Estrogens Used to Inhibit the Succinoxidase
System of Rat Liver Tissue

No. of expts.	Material injected*	Total dose estrogen γ	No. animals used	Per cent animals in estrus
Diethylstilbestrol				
2	Estrogen	1.5	24	66
4	Boiled tissue plus estrogen	1.5	34	80
3	Flask contents	1.5	28	75
1	Flask contents	15.0	8	100
Sodium estrone sulphate				
1	Estrogen	3.5	23	52
2	Boiled tissue plus estrogen	3.5 and 4	12	42
2	Flask contents	3.5 and 4	12	50

*The tissue control was prepared by heating the homogenate in a boiling water bath for 10 minutes and mixing with the proper amount of the fortified substrate. The experimental tissue was obtained from the Warburg flasks at the end of runs in which the estrogens were used as inhibitors.

by liver homogenates (Table XIV). It is to be emphasized that the media which we used for determining the activity of the succinoxidase system are not optimal for measuring estrogen inactivation (16, 17). It is also probable that the quantities of liver used in our studies were not enough to cause any appreciable inactivation of the estrogens.

In regard to the inactivation of estrogens by the liver the results of De Meio *et al.*, are of interest (16). They have suggested that the cytochrome-cytochrome oxidase system probably is not an important factor in the inactivation of estradiol, but that a dehydrogenase system (or systems) is involved. Therefore, it seems that the enzyme system which inactivates estrogens is not the same as the one which is inhibited by them.

The data presented in this paper provide the basis for postulating that if the concentration of certain phenolic estrogens were to rise to high values in the organism the tissues might be damaged by the antioxidant action of the estrogens. Even if the estrogens

did not reach a high concentration, their presence in small amounts probably would alter the metabolism of certain tissues.

Damaging amounts of estrogens, however, probably do not accumulate to any extent in the liver and other tissues because the tissues, particularly the liver, are provided with estrogen-inactivating and estrogen-conjugating enzyme systems, which probably act on the phenolic hydroxyl groups of the estrogens, thus reducing their antioxidant action on the liver. Since the liver has the detoxifying mechanisms so well developed it serves as the first line of defense in protecting the other tissues against damage.

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MECHANISM OF ACTION OF LIPOXIDASE

RALPH T. HOLMAN

*Department of Biochemistry and Nutrition,
Agricultural and Mechanical College of Texas*

THE PURPOSE of this communication is to summarize the information gained thus far pertinent to the mechanism of the action of lipoxidase. The enzyme's action will be discussed from the point of view of the composition of the enzyme, *in vitro* oxidation of its substrates and coupled oxidation, and *in vivo* changes in composition of the soybean during the period of germination. The lipoxidase preparation used in all of the studies which will be mentioned here was prepared at the Medical Nobel Institute from a large sample of Swedish soybeans (1). The product obtained was found to be homogeneous as judged by electrophoresis and by diffusion and ultra centrifugation studies made in the Physical Chemical Institute at Uppsala. The enzyme appears as minute colorless leaflets when crystallized by increasing ammonium sulfate concentration. The protein behaves as a globulin being insoluble in distilled water but soluble in dilute salt solutions. It precipitates at approximately 50% saturated ammonium sulfate. The absorption spectrum of pure lipoxidase shown in Figure 1 is that of a common protein showing only the usual absorption band at approximately 2800 Å. The ultraviolet absorption spectrum shows no evidence of other bands which could be due to prosthetic groups. In the course of the preparation of the enzyme it was dialyzed repeatedly and for long periods of time, and so any easily-dissociable prosthetic group or coenzymes would probably have been removed. The reaction is routinely carried out in a system containing only linoleate, borate buffer and lipoxidase (2). Thus the pure enzyme gives no evidence of requiring a cofactor for its action. Samples of impure lipoxidase have been ashed and iron has been determined upon ash. A preparation 94% pure, as judged by activity, had an iron content which would require a molecular weight of 370,000 for one atom of iron per molecule. However, this iron must be present as an impurity because the molecular

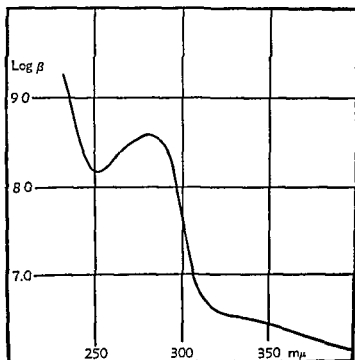


FIGURE 1. Ultraviolet Absorption Spectrum of Lipoxidase.

$$\text{In } \frac{I_0}{I} = \frac{c \cdot d}{\text{when } c = \text{moles/ml}}$$

weight as found by sedimentation experiments is 102,000. It thus appears that lipoxidase is not active through an iron atom in its molecule. This is substantiated by several inhibitor experiments. Lipoxidase has been found to be active in the presence of relatively high concentrations of cyanide, pyrophosphate, azide, fluoride and diethylthiocarbamic acid. Thus it is unlikely that lipoxidase acts through some heavy metal catalysis. No detectable sulfhydryl group has been found in the enzyme. The enzyme is also active in relatively high concentrations of parachloromercuribenzoate. This enzyme then appears to be unique in that it acts without a detectable prosthetic group and performs a function commonly catalyzed by heavy metals without the agency of the same.

Studies on the amino acid composition of lipoxidase indicate the presence of several of the common amino acids (Table 1). From this analysis it is impossible as yet to see what might be the active center in the enzyme molecule. One point of interest is that some unusual amino acid is present in this enzyme.

Competitive inhibition of lipoxidase action upon linoleic acid is exhibited by several fatty acids (3). *trans*-Linoleic acid, conjugated linoleic acid, oleic acid and octanoic acid exhibit competi-

TABLE I
Residues of Amino Acid Per Mole Lipoxidase

Alanine	60*	Methionine	13
Arginine	30	Phenylalanine	30
Aspartic Acid	47	Proline	46
Cystine	10*	Serine	80*
Glutamic Acid	73	Threonine	53
Glycine	82	Tryptophane	4
Histidine	22	Tyrosine	35
Hydroxylysine	35*	Valine	65
iso-Leucine	63	Unknown 1	110*
Leucine	89	Unknown 2	50*
Lysine	54		

*Indicated by paper chromatogram

tive attraction for the enzyme's active center in decreasing order. The only effective inhibitors for lipoxidase action which have thus far been found are alpha-naphthol and alpha-tocopherol. It thus appears at this early stage of the investigation that the most effective inhibitors are the antioxidants.

By way of discussion of the action of lipoxidase it must be explained that in all the tests for lipoxidase action and for its assay, the spectrophotometric assay developed by Theorell, *et al.*, (2) has been used. Essentially this reaction medium is as follows: 2 ml of linoleic acid in 1 ml borate buffer at pH 9 are subjected to the action of the appropriate quantity of lipoxidase under an atmosphere of oxygen. At the end of 2 minutes the reaction is stopped by the addition of alcohol, the solution is diluted appropriately and the light absorption at 2340 Å is measured. The extinction at this wavelength is a measure of the degree of conjugation of the double bonds of linoleic acid as a consequence of its oxidation. In this homogeneous system no activator is needed for the system as was the case in the emulsion systems used by many other investigators. The reaction proceeds linearly over long periods of time at low temperatures in this simple system, whereas even at room temperature the reaction ceases much earlier. Apparently the inactivation of the enzyme as a consequence of its action is thermally sensitive. The conjugation induced by the lipoxidase action is virtually complete at 0°C (3,4). The molar extinction coefficient at 2340 Å of partially oxidized samples of linoleate based upon the oxygen uptake was found to be 31,400 at 0°, but

this decreased to 23,000 at 37°C. The absorption at 2700 Å, due to side products, increased considerably at the higher temperature, indicating that perhaps the inactivation at higher temperatures may be due to side products.

The conditions under which lipoxidase acts in the studies reported here are quite different from physiological conditions. The homogeneous solution at pH 9 was chosen to eliminate difficulties involved with emulsions and to make the substrate available to the enzyme. Thus, the optimum pH for *overall* activity was found to be above pH 9. This undoubtedly is largely due to the accessibility of the substrate to the enzyme. These results should not be interpreted as indicating the optimum pH under physiological conditions. They merely indicate that the product of the enzyme activity and effective concentration of the substrate

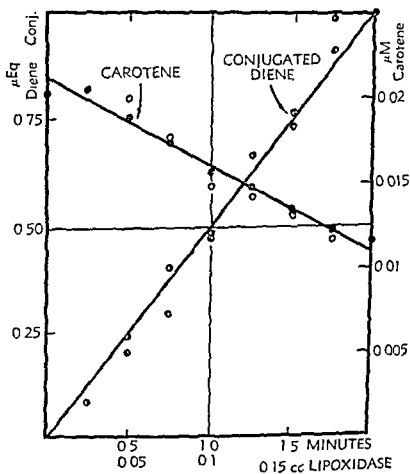


FIGURE 2. The course of lipoxidase action upon sodium linoleate containing carotene.

reaches a maximum above pH 9.

A study of the coupled oxidation of such easily-oxidizable substances as carotenoids has given some information regarding lipoxidase action (5). In Figure 2 the relationships between the time of reaction and diene conjugation, time of reaction and carotene destruction, enzyme concentration and carotene destruction as well as diene conjugation, and enzyme concentration and carotene destruction are all shown. It appears that carotene destruction as well as diene conjugation can be used as a measure of lipoxidase concentration, using a two-minute reaction period. It also appears that the production of diene conjugation and the destruction of carotene are approximately linear with time.

In Figure 3 is shown the effect of increasing carotenoid concentration in the reaction mixture. As carotenoid is increased, the degree of diene conjugation is decreased and the quantity of

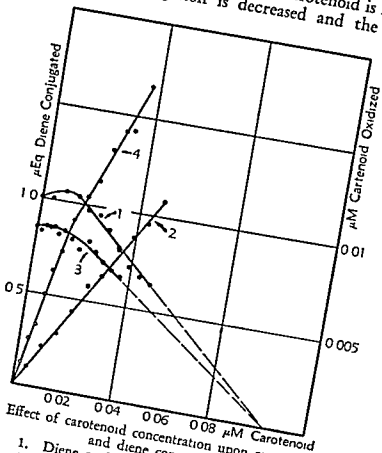


FIGURE 3. Effect of carotenoid concentration upon carotenoid destruction and diene conjugation.

1. Diene conjugation vs. carotene concentration
2. Carotene destroyed vs. carotene concentration
3. Diene conjugation vs. bixin concentration
4. Bixin destruction vs. bixin concentration

carotenoid oxidized is increased. It may be that the carotenoid oxidation occurs as a consequence of its interruption of some chain mechanism. The number of dienes not conjugated as a consequence of the oxidation of one carotenoid molecule are approximately 43 and 26 for carotene and bixin respectively. This may mean that under these conditions the effective chain lengths are 43 and 26 respectively.

In Figure 4 we have the course of the reaction of lipoxidase and linoleic acid in the presence of bixin as automatically recorded with a recording spectrophotometer by Dr. Britton Chance. This apparatus allows the mixture of two solutions in a small capillary reaction vessel by the simultaneous discharge of a pair of syringes. The change in absorption spectrum at 4000 Å and the oxygen tension were measured and recorded electrically. From the results obtained with this apparatus it appears that lipoxidase oxidation of linoleate does have an induction period. It will be noted that the destruction of bixin and oxygen consumption proceed after a short but definite lag phase. The second portion of the figure was taken under the same conditions as the first except that one-fifth as much enzyme was used. In the third portion the conditions are the same as the second except that one-half as much bixin

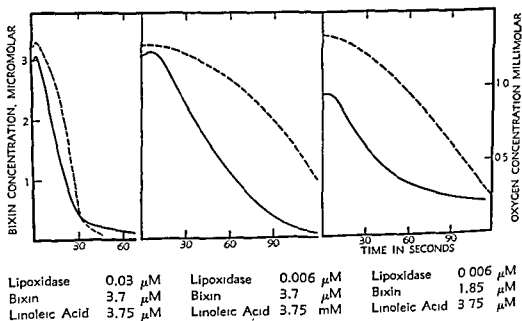


FIGURE 4. Coupled oxidation of Bixin in sodium linoleate oxidized with lipoxidase recorded electrically.

--- oxygen uptake
 — Bixin concentration

was used. The exact meaning of these data are not known, but it should be pointed out that even enzymatic linoleate oxidation has an induction period. This may be taken as evidence for an autocatalytic oxidation of linoleate initiated by lipoxidase.

A single experiment has been performed in which the effect of increasing tocopherol concentration upon apparent diene conjugation has been studied. From the results shown in Figure 5 it again appears that diene conjugation was inhibited by the presence of tocopherol. However, tocopherol as measured by its absorption at 2900 Å was not destroyed during this reaction. It may well be that the tocopherol concentration was too high to measure adequately the tocopherol destruction. It may also be that tocopherol is destroyed far less easily than is bixin or carotene.

In a series of experiments on the changes in lipoxidase concentration and the changes in fat composition in the germinating soybean (6), some interesting observations have been made, and

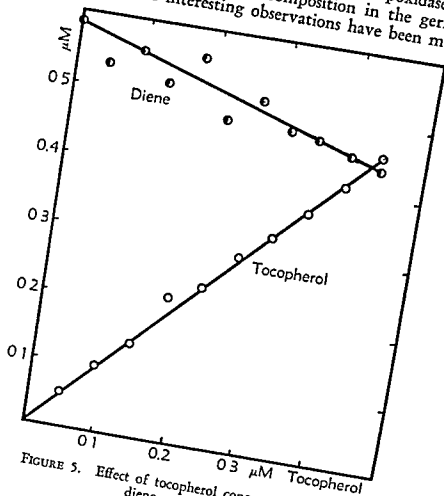


FIGURE 5. Effect of tocopherol concentrations upon diene conjugation.

are shown in Figure 6. As is well known during the germination phase of the plant's life, its total fat was found to decrease. Iodine number was also found to decrease and linoleic and linolenic acid concentrations in the soybean oil decreased during this phase. Contrary to expectation, however, lipoxidase activity decreased during this phase. At the time when lipoxidase decreases rapidly, catalase concentration increases sharply to a maximum and then decreases sharply. Some connection between catalase activity and fat metabolism has been suspected by workers in this field. It has been found, for example, that catalase is easily prepared in good yield from extracts of adipose tissue (7). Unpublished experiments have demonstrated that there probably is a complex formation between linoleate peroxide and catalase as measured in the recording spectrophotometer by Dr. Chance. A shift in absorption spectrum upon the mixture of linoleate, lipoxidase and catalase takes place indicating some binding of catalase. This shift does not take place with other combinations of these reactants. However, the complex is not dissociable in the presence of oxidizable substrates as is the case with catalase, hydrogen peroxide and ethyl alcohol.

From the study of germination, it appears that lipoxidase activity is high in the dormant bean, that the activity decreases markedly at the time when the enzyme's activity takes effect. Perhaps the enzyme is protected by the relatively dehydrated condition prevailing in the tissue during dormancy and that the reaction is initiated when the bean absorbs water prior to germination. It also appears that the enzyme is not needed in its original concentration to perpetuate the oxidation of the naturally-occurring substrates, linoleate and linolenate. Thus, *in vivo*, the enzyme's action may be that of initiator of the chain autoxidation of these substances.

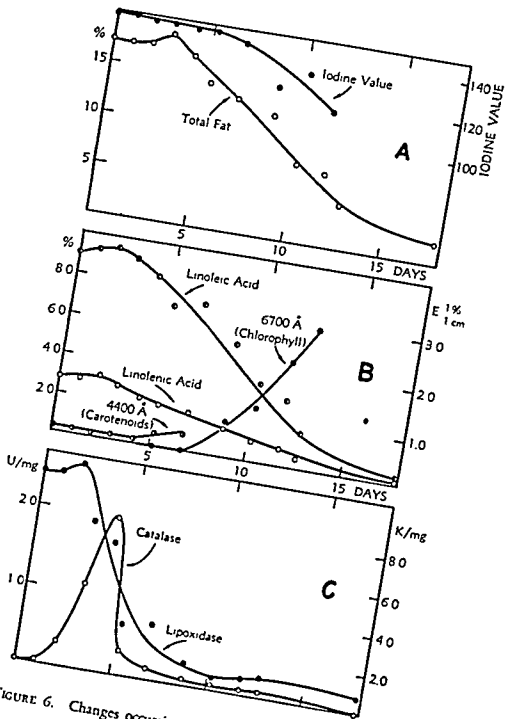


FIGURE 6. Changes occurring in the germinating soybean.

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DISCUSSION

Lundberg: The paper given by Dr. Holman has raised once again the question whether the biological oxidation of unsaturated fatty acids may involve a chain reaction in the classical physico-chemical sense. It must be agreed that Dr. Holman's observation that the destruction of a single carotene molecule is associated with the prevention of the formation of a large number of linoleate peroxide molecules is suggestive of a possible chain reaction. This particular observation is entirely consistent with the hypothesis that the lipoxidase molecule may serve to initiate a reaction chain by producing a free radical and that the peroxide molecules are then formed in a sequence of reactions in the same manner as proposed by Bolland and his coworkers for the thermal oxidation of pure ethyl linoleate.

As in previous conferences, I again wish to take the position that while it may yet be shown that the biological oxidation of fatty acids in systems such as the one described by Dr. Holman does occur by means of a chain reaction, conclusive proof of such a mechanism is still definitely lacking. Going further, it seems to me that there are two good reasons why the classical type of chain mechanism should not yet be accepted even as a working hypothesis in these biological systems: (1) In the present hazy state of our knowledge of the mechanisms of enzyme reactions, there appear to be several other mechanisms that could account for Dr. Holman's results equally as well as a chain mechanism. (Most postulated enzyme mechanisms exhibit a kind of chain character

but they do not conform to the physico-chemical concept of a chain reaction, since they involve intermediary substances.) Since these other mechanisms are entirely speculative, we shall not consider them further at this time.

(2) There are data available which strongly suggest that the mechanisms of the thermal oxidation and of the lipoxidase-catalyzed oxidation of linoleates may be distinctly different. It is evidence of this character that I should like to introduce into the present discussion.

It is now well-known that in the oxidation of unconjugated polyenoic substances such as linoleates and linolenates, not only does one obtain peroxides but there may occur also a shifting of some double bonds so that some conjugated substances are formed.

Bergstrom and Holman studied the lipoxidase-catalyzed oxidation of a linoleate at 0°C . and obtained good spectrophotometric evidence that the peroxides formed under these conditions are 100% conjugated. On the other hand, in the thermal oxidation of methyl linoleate at 0°C . only about 70% of the peroxide molecules are produced in a conjugated state. In this connection it should be pointed out that Bolland and Gee calculated on the basis of bond energies and resonance energies that a conjugated diene hydroperoxide is more stable than an unconjugated diene hydroperoxide.

Since peroxides other than hydroperoxides are thermodynamically and kinetically possible, as a result of secondary reactions, it would be desirable to show that in the thermal oxidation of linoleate at 0° , the unconjugated peroxides formed are primarily unconjugated monomeric hydroperoxides, and therefore are presumably formed not in secondary reactions but, as postulated by Bolland, are formed in the same chain mechanism that is involved in the production of the conjugated hydroperoxides. The formation of the less stable unconjugated monomeric hydroperoxides in the thermal oxidation at 0°C ., and the failure to obtain these same forms in the lipoxidase-catalyzed oxidation of 0° would indicate quite different mechanisms in the two cases.

There has been much confusion in the literature concerning the structure of the peroxides that are formed in the thermal oxidation of linoleates. Bolland and Gee have calculated that in addition to the simple monomeric hydroperoxides, various cyclic and polymeric peroxides are thermodynamically and kinetically possible. Furthermore, Bergstrom obtained chemical evidence which suggested the absence of the unconjugated 11-hydroperoxide in oxidized methyl linoleate, even though the 11-hydroperoxide would be the unconjugated hydroperoxide that would be expected

on the basis of the formulations of Farmer, Bolland, and their coworkers.

Various attempts have been made in the past to determine the structures of the oxidation products of fatty acids and their esters by the application of ordinary fat analytical methods directly to the oxidation products. Such attempts have failed for two reasons: (1) the application of many of these analytical methods requires a relatively high concentration of the oxidation products. It is not possible to obtain a high concentration of the initial oxidation products directly by oxidation without developing a wide variety of secondary products which make it impossible to interpret the analytical results; (2) many of the fat analytical methods yield false values when applied to oxidized fats because of the interference of the peroxide groups with the accuracy of the determinations.

Recently we have obtained a better insight by indirect chemical methods into the structure of the peroxides formed initially in the oxidation of methyl linoleate. In our recent studies of the thermal oxidation of methyl linoleate we have overcome the difficulties just mentioned by two methods: (1) we have restricted the oxidation of methyl linoleate to a relatively low level of oxygen uptake in order to minimize the production of secondary products, and then have been able to concentrate the peroxides without evidence of chemical alteration; (2) we have found that the peroxide concentrate in an acetic acid-chloroform solution may be reduced with potassium iodide without appreciably affecting the double bond structure that was present before the reduction.

Without going into all of the experimental details, I shall summarize the results briefly. First, it was found that the reduction of the peroxide concentrate did not significantly alter the spectral absorption in the region of 2325 \AA , thus indicating that the conjugated centers were unaffected by the reduction. Secondly, the Woburn iodine value for the reduced concentrate corresponded to two double bonds per mol, indicating that virtually no double bonds had been destroyed during the formation of the peroxide. Third, it was found that the hydroxyl content of the reduced compound corresponded closely with the anticipated value if it was assumed that one hydroperoxide on reduction yields one hydroxyl group. Fourth, the alpha-glycolic value of the reduced compound was very low, suggesting that the peroxide concentrate had a very low content of cyclic peroxides. Finally, the molecular weights of the peroxide concentrate and the reduced concentrate corresponded very closely with the calculated values for a pure

monomeric monoperoxide and methyl hydroxy-octadecadienoate, respectively. From these results, it may be concluded that virtually all the peroxides formed during the early stages of the thermal oxidation of methyl linoleate were monomeric monohydroperoxides. Since only a portion of these were present as conjugated hydroperoxides, it must be concluded that the remainder consisted of unconjugated monomeric monohydroperoxides.

Since these less stable unconjugated hydroperoxides are not formed in the lipoxidase-catalyzed oxidation at 0°C., it would seem to be indicated that in this case the lipoxidase does not act by simply starting a reaction chain, and that some other mechanism must be involved in the lipoxidase-catalyzed reaction.

However, there is one valid objection that may be raised at this point. In our experiments, the methyl linoleate was oxidized in pure form, whereas in Holman's system, the linoleate was present as a soap in a water solution. As an incomplete answer to this objection, I may say that we have thermally oxidized sodium linoleate in water solution at approximately 20°C. and although we have not conducted a complete series of analyses on the products as in the case of methyl linoleate peroxides, we have measured the spectral absorption at 2325 Å and have found it to be lower than is obtained in the oxidation of methyl linoleate at 20°C. and considerably lower than would be obtained if conjugated diene hydroperoxides were formed exclusively.

Thus from these observations one may speculate that the lipoxidase may form a complex with each linoleate molecule before it is oxidized, in such a way as to block the formation of an 11-hydroperoxide and at the same time directing the oxygen to the 9 or 13 positions. Such a reaction, of course, may involve free radicals but would not necessarily occur by a chain mechanism of the classical physico-chemical type.

Michaelis: I do not believe there are any chain reactions in living organisms in the sense that a free radical should start a chain by free collision with molecules, such as the reaction $H_2 + Cl_2 \rightarrow 2HCl$, which is primed by free chlorine atoms and proceeds as a succession of binary collision reactions. In the living organism (and even in relatively simple catalytic oxidative systems, such as the autoxidation of cysteine with iron as catalyst), the essential part of what may be called a "chain reaction" occurs within the substrate-catalyst compound. A simple, free collision would not explain the specificity of enzymatic reactions.

Tobolsky: As you know, Bolland, Cree and Farmer have stated that the exclusive initial product of the oxidation of ethyl linole-

Holman: I do not know if lipoxidase occurs in the castor bean, but there is much enzyme in all of the legumes that have been tested.

Ames: In the few remaining minutes, I would like to amplify the data on partition chromatography of lipoxidase hydrolysates, as presented by Dr. Holman. First, I would like to show you a copy of a key to the location of the ninhydrin reacting materials on a two-dimensional chromatogram.

We have run both acid and alkaline hydrolysates of lipoxidase, using the technique of paper partition chromatography. Quite a number of the common amino acids are present in the lipoxidase molecule. The presence of such amino acids as glutamic acid, alanine, glycine, valine, tyrosine, the leucines and phenylalanine is easily shown. On the other hand, such common amino acids as aspartic acid, proline, tyrosine and tryptophane are not indicated, and cystine and methionine cannot be demonstrated with certainty in the hydrolysates. There are several spots on the two-dimensional chromatogram which have not as yet been identified. The final results will be extremely interesting since not only will we have an estimation of the amino acid content of a highly purified enzyme, but, also, because we will have a direct comparison between chromatographic and microbiological techniques.

Holman: We are checking some of these findings microbiologically.

Rusch: The level of lipoxidase in the germinating soybeans may have a counterpart in the developing grasshopper egg. Bodine and his associates (*J. Cell. & Comp. Physiol.* 14, 173, 1939) reported that the lipids of these eggs decreased most rapidly during the stages of greatest cellular proliferation and differentiation. It would be very interesting to know the level of lipoxidase at the various stages of embryonic development in these eggs.

